Role of Müller cells in the onset and progression of neuronal degeneration after acute retinal ischemia

Dissertation

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List of abbreviations

BBB *blood-brain barrier*

BDNF *brain-derived neurotrophic factor*

bFGF *basic fibroblast growth factor*

BRB *blood-retina barrier*

CNS *central nervous system*

CNTF *ciliary neurotrophic factor*

DBI *diazepam binding inhibitor*

FC *fluorocitrate*

FXN *frataxin*

GCL *ganglion cell layer*

GDNF *glial cell line-derived neurotrophic factor*

Gpx1 *glutathione peroxidase 1*

INL *inner nuclear layer*

IOP *intraocular pressure*

IPL *inner plexiform layer*

LIF *leukemia inhibitory factor*

MCAO *middle cerebral artery occlusion*

NFL *nerve fibre layer*

NGF *nerve growth factor*

ON *optic nerve*

ONL *outer nuclear layer*

RGCs *retinal ganglion cells*

ROS *reactive oxygen species*

RPE *retinal pigment epithelium*

Sod2 *superoxide dismutase*

TNF-α *tumor necrosis factor-alpha*

VEGF *vascular endothelial growth factor*

VMD *vitelliform macular dystrophy*

1 Summary

Reactive gliosis is an early pathological feature common to most neurodegenerative diseases and retinal pathologies. Under physiological conditions, Müller cells, the most abundant glia cell type of the retina, maintain a critical retinal homeostatic balance. After stress or retinal injury, Müller cells become activated and perform a variety of tasks including recycling of neurotoxic glutamate, formation of the glia scar to inhibit lesion spreading, restoration of the blood supply, and inhibition of neuronal apoptosis by growth factor secretion and restoration of the blood-retinal barrier. However, the Müller cell response can also result in vascular leakage, pathologic neovascularization and reduced axonal regrowth, which is detrimental for neuronal survival and tissue recovery. This “Janus face”-like behavior of reactive glia cells has been the focus of extensive research, yet its impact and timely regulation remain poorly understood.

The aim of this study was to evaluate the timing for the transition in the Müller cell gliotic response from neuroprotective to detrimental and the effect of an enhanced antioxidant activity in these cells on neuronal survival after an acute ischemia/reperfusion injury *in vivo*. Neuronal degeneration was induced by elevating the intraocular pressure above systolic pressure leading to retinal ischemia.

To analyze the temporal pattern of the Müller cell gliotic response during and after ischemia, the glial toxin fluorocitrate was injected into the eye vitreous leading to specific inhibition of the Krebs cycle in Müller cells. The Müller cell metabolism was impaired at different time points before and after lesion onset. Transient Müller cell inhibition allowed the identification of three phases in the Müller cell gliotic response: first, early at the onset of the retinal lesion, reactive Müller cells are critical for neuronal survival. Metabolic inhibition during this phase is detrimental for neuron survival. Second, during the acute phase after lesion and reperfusion onset, Müllers cells seem not to play a critical role in neuronal survival, because impaired metabolism did not affect the neuronal cell death. Third, during the sub-chronic phase starting 12-18 hours after lesion, inhibition of the cell metabolism led to increased neuronal survival. This time frame comprises the transition from a neuroprotective to a detrimental response after an ischemic injury. Therefore, the gliotic response after ischemia can be divided in an early first neuroprotective phase followed by a transition phase and a later detrimental phase.

One hallmark of ischemia is the increased level of oxidative stress, in particular during the reperfusion phase after lesion. Therefore, in the second part of the study, we evaluated the

effect of a selective increase of the antioxidant response of Müller cells on neuronal survival after ischemia. This was achieved by overexpression of the mitochondrial enzyme frataxin in these cells. After ischemia, the neuronal survival was significantly increased in frataxin overexpressing mice. This was accompanaied by an increase in the expression of antioxidative enzymes. This finding supports the notion that frataxin is neuroprotective by decreasing oxidative stress after an ischemia/reperfusion injury. Interestingly, Müller cells themselves did not show signs of an altered gliotic response, as indicated by unchanged expression levels of intermediary filaments after lesion. Frataxin expression was also associated with an increased expression of neurotrophic factors. Furthermore, support of the Müller cell function after lesion led to a reduced reactivity of retinal microglia, the main cell type involved in the inflammatory response after lesion.

The two approaches used in this study show that the time-dependent modulation of the response of Müller cells and also their functional support leads to an increase in neuronal survival after injury, which is associated with a reduced glial detrimental response and enhanced intrinsic neuron-supportive mechanisms.

Thus, modulation of the Müller cell response to lesion renders these cells a feasible target to improve both retinal homeostasis and the neuronal outcome after an acute ischemic lesion of the retina. Furthermore, our study supports the notion that glial cells might represent a critical target for the development of strategies aimed to reduce the neuronal cell loss caused by neurodegenerative pathologies and after lesion in the central nervous system.

2 Zusammenfassung

Reaktive Gliose ist ein frühes pathologisches Merkmal der meisten neurodegenerativen Erkrankungen und Netzhautpathologien. Müllerzellen, der häufigste Glia-Zelltyp der Netzhaut, sind unter physiologischen Bedingungen für die Aufrechterhaltung eines homöostatischen Gleichgewichtes der Retina verantwortlich. Nach Stress oder Verletzungen an der Netzhaut werden Müllerzellen aktiviert und sind, unter anderem, für die Bildung der Glia-Narbe (Unterbindung der Ausweitung der Schädigung), für die Wiederherstellung der Blutversorgung und der Blut-Retina Schranke sowie für die Hemmung der neuronalen Apoptose durch die Sekretion von Wachstumsfaktoren verantwortlich. Die Müllerzell-Antwort kann jedoch auch zu einer erhöhten Durchlässigkeit von Blutgefäßen, pathologischer Neovaskularisation und einem reduziertem Nachwachsen der Axone führen, was für das neuronale Überleben und die Geweberegeneration nachteilig ist. Dieses „doppelgesichtige“ Verhalten von reaktiven Gliazellen liegt im Fokus umfangreicher Forschungen, doch deren Wirkung und zeitliche Regulierung sind noch nicht vollständig geklärt.

Das Ziel dieser Studie war es, den Zeitpunkt für den Übergang der gliotischen Müllerzell-Antwort von neuroprotektiv zu schädlich, und die Wirkung einer verstärkten antioxidativen Aktivität dieser Zellen auf das neuronale Überleben nach einer akuten ischämischen Reperfusions-Schädigung *in vivo* zu untersuchen. Die neuronale Degeneration wurde hierfür durch die Erhöhung des Augeninnendrucks über den systolischen Druck induziert, was zu einer retinalen Ischämie führte.

Für die Untersuchung des zeitlichen Ablaufs der gliotischen Antwort von Müllerzellen während und nach der Ischämie wurde das Glia-Toxin Fluorocitrat in den Glaskörper des Auges injiziert. Dies führte zu einer spezifischen Hemmung des Krebszyklus in Müllerzellen und somit zu einem reduzierten Zellmetabolismus. Der Stoffwechsel der Müllerzellen konnte dadurch zu verschiedenen Zeitpunkten vor und nach Beginn der Ischämie unterbunden werden. Die transiente Hemmung der Müllerzellen ermöglichte die Identifizierung von drei Phasen der gliotischen Zellantwort. Frühzeitig zu Beginn der retinalen Läsion sind reaktive Müllerzellen entscheidend für das Überleben der Neurone. Eine metabolische Hemmung während dieser Phase wirkt sich nachteilig auf das Überleben der retinalen Ganglienzellen aus. Während des Fortschreitens der Läsion scheinen Müllerzellen keine kritische Rolle für das neuronale Überleben zu spielen, da ein gestörter Müllerzell-Metabolismus den neuronalen Zelltod nicht beeinflusst. Die Hemmung des Zellmetabolismus während der subchronischen Phase, beginnend 12-18 Stunden nach der

Läsion, führt jedoch zu einem erhöhten Überleben der Neurone. Zu diesem Zeitpunkt erfolgt der Übergang von einer neuroprotektiven zu einer Neuron-schädlichen Zellantwort nach einer ischämischen Verletzung. Daher kann die gliotische Zellantwort in drei Hauptphasen eingeteilt werden: eine frühe neuroprotektive Phase, gefolgt vom Übergang zu einer späteren schädlichen Phase.

Ein charakteristisches Merkmal für die Folgen einer Ischämie ist die Erhöhung des oxidativen Stresses, die insbesondere während der Reperfusionsphase nach der Läsion stattfindet. Daher wurde im zweiten Teil der Studie die Wirkung einer selektiven Erhöhung der antioxidativen Antwort der Müllerzellen auf das neuronale Überleben nach Ischämie untersucht. Dies wurde durch Überexpression des mitochondrialen Enzyms Frataxin in Müllerzellen erreicht. Nach Ischämie war in Frataxin-überexprimierenden Mäusen das neuronale Überleben retinaler Ganglienzellen signifikant erhöht, das zudem mit einer erhöhten Expression von antioxidativen Enzymen korrelierte. Dieses Ergebnis stützt die Annahme, dass Frataxin neuroprotektiv wirkt indem es den oxidativen Stress nach einer ischämischen Reperfusionsverletzung verringert. Interessanterweise zeigten die Müllerzellen selbst keine Anzeichen einer veränderten gliotischen Antwort, was anhand eines unveränderten Expressionsverhaltens von Intermediärfilamenten nach der Läsion erkennbar war. Des Weiteren war die Unterstützung der Müllerzell-Funktion mit einer gesteigerten Expression neurotropher Faktoren assoziiert. Zusätzlich führte dies zu einer verminderten Reaktivität der retinalen Mikrogliazellen, dem Zelltyp, der an der Entzündungsreaktion nach der Läsion beteiligt ist.

Die beiden in dieser Studie verwendeten Ansätze zur Modulation der gliotischen Müllerzellantwort zeigen, dass eine zeitabhängige Beeinflussung oder eine funktionelle Unterstützung der Müllerzellen zu einer Erhöhung des neuronalen Überlebens nach einer retinalen Verletzung führen. Dies geht einher mit einer verminderten Neuron-schädlichen Gliazell-Antwort und einer Verstärkung der intrinsischen unterstützenden Mechanismen für retinale Ganglienzellen.

Die Möglichkeit der Modulation der Müllerzell-Antwort macht diese Zellen zu einem lukrativen Ziel zur Verbesserung der retinalen Homöostase und des neuronalen Überlebens nach einer akuten retinalen Ischämie. Darüber hinaus unterstützt unsere Studie die Annahme, dass Gliazellen ein kritisches Ziel für die Entwicklung von Strategien darstellen könnten, die darauf abzielen, den durch neurodegenerative Pathologien und nach Läsionen im Zentralnervensystem verursachten neuronalen Zellverlust zu reduzieren.

3 Introduction

3.1 The central nervous system and the retina

The central nervous system (CNS) integrates information that it receives to coordinate and influence the activity of all body parts of all multicellular animals. From insects to mammals, the CNS comprises a network of between 100.000 (fruit fly) and 10^{14} (human) neurons which mediate information transmission and processing. However, neurons are not the only cell type of the CNS. Neurons represent only half of all brain cells in mammals, although the proportion varies between different brain regions. The rest of the brain cells consist of glial cells (von Bartheld *et al.* 2016). Glial cells were firstly described by Rudolf Virchow (1821 – 1902), a German pathologist who named the cells "neuroglia", from Greek for "nerve glue". At that time, Virchow assumed that these cells, which are obviously not neurons, represented the connective tissue of the brain (Virchow 1858). More recently, however, two major roles have been assigned to this cell type, including neuronal support and tissue repair. Intense research during the past 30 years indicates that glia cells play an active role during development and in many central homeostatic processes. Neuroglias found in the CNS include astrocytes, oligodendrocytes, NG2-glia and ependymal cells. All these cells in the CNS are referred to either as macroglia or microglia, whereas the cells in the peripheral nervous system are known as Schwann cells or satellite glial cells.

The neural retina is anatomically and developmentally an extension of the brain. It comprises highly specialized cell types, which generate visual information and transmitt them between the eye and the brain. During development, the retina and optic nerve extend from the diencephalon. The first retinal cells to be born are ganglion cells, which are the only neurons to project axons to the brain. These are followed by horizontal cells, cones and displaced amacrine cells. Later, amacrine cells, bipolar cells, rod cells and Müller cells are generated, with Müller cells being the last cells to become post-mitotic (Young 1985). Retinal cells form a 9-layered structure, with their cell nuclei and somas located in nuclear layers and the ganglion cell layer (GCL), and cellular extensions and synapses located in the plexiform layers and the nerve fibre layer (NFL). The sensory components of the retina are made of two types of photoreceptors, rods and cones. The photoreceptor outer segments are located in the retinal pigment epithelium (RPE), and the somas reside in the outer nuclear layer (ONL). Signals from photoreceptors are conveyed by interneurons comprising bipolar- horizontal- and amacrine cells which are found in the inner nuclear layer (INL) and

further project to retinal ganglion cells (RGCs) in the GCL. The axons of the RGCs form the optic nerve (ON) and transmit the visual information to the visual cortex in the brain.

3.2 Retinal glial cells

In the adult retina (figure 1), the glial population consists of two macroglial cell types (astrocytes and Müller cells) and the microglia. Müller cells form the structural scaffolding of the retina and span the entire retinal thickness. Astrocytes reside in the NFL and their processes project in the GCL/inner plexiform layer (IPL). Microglia are distributed in the plexiform layers, GCL and NFL (Bosco *et al.* 2011; Chen *et al.* 2002).

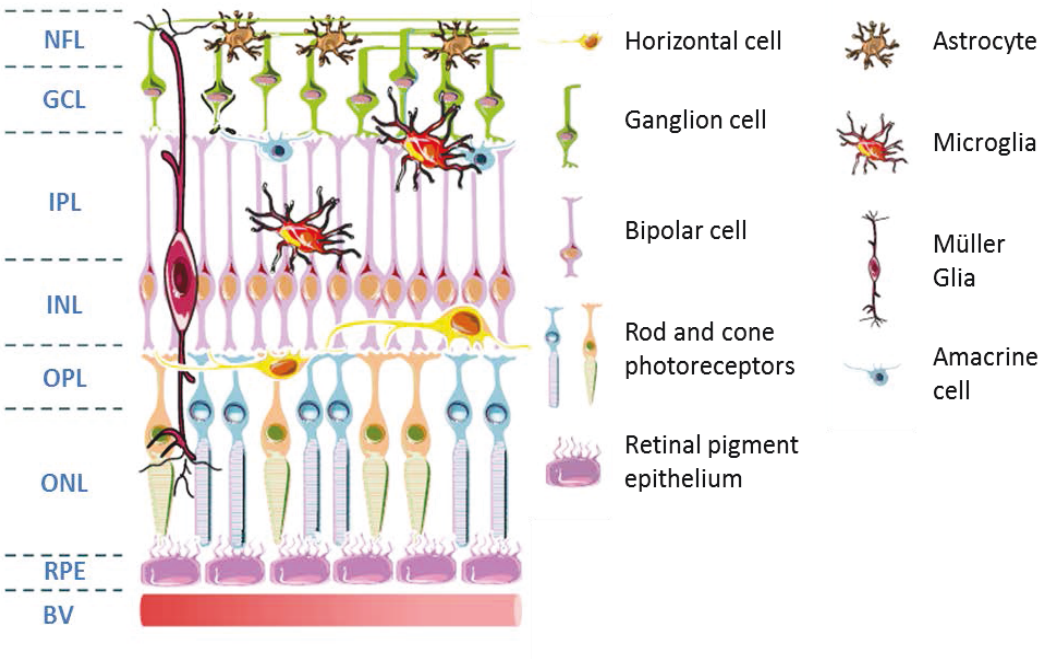

Figure 1: Schematic representation of the retinal layers and cellular components of the retina including neuronal, glial and sensory cell types. NFL = nerve fibre layer, GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, RPE = retinal pigment epithelium; BV = choroidal blood vessels, modified after Boia et al. (2015).

Astrocytes enter the developing retina from the brain along the optic nerve together with blood vessels and migrate into the retina through the optic nerve head. They spread towards the peripheral margins of the retina but remain restricted to the GCL/NFL. Astrocytes have a characteristic stellar-like morphology with a flattened cell body and a series of fibrous radiating processes branching deeper into the retinal layers, ensheathing blood vessels and axons (Stone and Dreher 1987; Huxlin *et al.* 1992; Chan-Ling 1994; Kur *et*

al. 2012; Vecino *et al.* 2015). As the main producer of VEGF (vascular endothelial growth factor), astrocytes offer developmental support for the retinal vasculature during both normal (Stone *et al.* 1995) and pathological (Ozaki *et al.* 2000) vessel formation.

Müller cells are the most abundant glial cell type in the vertebrate retina and are homogeneously distributed in this tissue (Wang et al., 2016). They have a bipolar morphology with somas residing in the INL, from which radially oriented processes emerge, extending from the inner limiting membrane to the outer limiting membrane. Müller cells extend apical microvilli into the sub-retinal space between the inner segments of photoreceptor cells, whereas on the opposite side, the inner processes approach the vitreal surface forming endfeet that border the basal lamina between the vitreous body and the neuroretina. In the nuclear layers, the lamellar processes of Müller cells form structures that envelop the cell bodies of neuronal cells where they support synaptic functions similar to those of brain protoplasmic astrocytes (Rasmussen 1972; Hama *et al.* 1978; Reichenbach *et al.* 1989; Clarke and Barres 2013; Reichenbach and Bringmann 2013; Allen 2014; Vecino *et al.* 2015). Müller cells have overlapping functions with astrocytes and are important for retinal cell and tissue homeostasis, including regulation of the K+ and water content, secretion of trophic factors, removal of metabolic waste, and neurotransmitter recycling. Müller cells, together with astrocytes, are essential for the organization and maintenance of the blood–retina barrier (BRB). Müller cells participate in the visual cycle and act as optic fibers guiding light through the retina (Ransom and Orkand 1996; Newman and Reichenbach 1996; Wang and Kefalov 2009; Wang and Kefalov 2011; Reichenbach and Bringmann 2013). Furthermore, similar to astrocytes in the brain, Müller cells provide metabolic support to surrounding retinal cells. They obtain glucose from the circulation to produce lactate, the primary metabolite used by retinal cells to produce ATP. Müller cells convert most of the glucose aerobically into lactate, instead of carbon dioxide and water, by oxidative phosphorylation via the so-called Warburg effect (Poitry-Yamate *et al.* 1995; Casson *et al.* 2012). Müller cells also accumulate glucose in internal cellular glycogen stores (Dringen et al. 1993; Kuwabara et al. 1961). Under metabolic stress, retinal Müller cells can provide metabolic support to retinal neurons by activating anaerobic glycolysis thereby oxidizing alternative substrates, such as lactate, glutamate or glutamine (Winkler 1981; Winkler *et al.* 2000; Winkler *et al.* 2003; Stone *et al.* 1999; Reichenbach and Bringmann 2010; Kitano *et al.* 1996). In addition to metabolic support, during stress, Müller cells also provide neurons with antioxidants such as glutathione (Schütte and Werner 1998).

Microglia are resident macrophages of the retina. They are long-lived cells predominantly maintained via self-renewal under steady-state conditions. Microglia have a ramified morphology with small somas and extensive branching of the dynamic processes. They are involved in active surveillance of their surrounding microenvironment (Nimmerjahn *et al.* 2005). Microglia in the normal and healthy tissue are maintained in a quiescent state but play an important role in supporting tissue and cell homeostasis. Furthermore, microglia constitute the first line of defense in CNS pathologies and become activated by various degenerative diseases and regulatory factors, thereby removing the damaged cells by phagocytosis (Dheen *et al.* 2007).

3.3 Reactive gliosis

Glia cells play a key role in the brain and in the retinal response to injury. They react to homeostatic changes by altering their morphology and function. Glia cells are able to withstand most insults, in contrast to the extremely vulnerable neurons (Anderson and Davis 1975; Schmuck *et al.* 2002; Winkler *et al.* 2000). Thus, glia cells take part in pathogenic processes and critically influence the neuronal fate. Müller cells, as well as astrocytes in the brain, show a “Janus face” like behavior after lesion. Gliotic alterations of Müller cells protect neuronal cells and retinal tissue, but also contribute to neuronal degeneration and edema development in the diseased retina (Bringmann *et al.* 2006; Bringmann *et al.* 2004). For instance, neuroprotective growth factors including glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), are either released by Müller cells or exert their effects indirectly by activating Müller cells (Rhee *et al.* 2013; Kirsch *et al.* 1997; Hauck *et al.* 2006; Wahlin *et al.* 2000; Wang *et al.* 2011). However, Müller cells also release VEGF which can be both neuroprotective by means of restoring blood supply and inhibiting apoptosis at early stages of reperfusion, and detrimental by inducing leakage and neovascularization in chronic diseases (Croll *et al.* 2004; Yasuhara *et al.* 2004; Kilic *et al.* 2006; Pierce *et al.* 1995; Tolentino *et al.* 2002). Glial scar formation by activated glial cells protects unlesioned neighboring tissue by restoring the blood-brain barrier (BBB) and the BRB integrity, restricting the inflammatory response and, thereby, limiting cellular degeneration (Faulkner *et al.* 2004). However, the glial scar also prevents neuronal recovery and impedes repair and remodeling of the neuronal tissue. Reactive glia secrete a number of extracellular matrix substrates known to inhibit dendritic and axonal growth (Shen *et al.* 2008; Moreau-Fauvarque *et al.* 2003). Moreover, Müller cells mediate cytotoxicity by releasing soluble factors such as the proinflammatory cytokine TNF-α (tumor necrosis

factor-alpha) which activates TNF-R1 (tumor necrosis factor-receptor 1) and triggers apoptosis (Cotinet *et al.* 1997; Tezel *et al.* 2001).

Beside Müller cells and astrocytes, microglia are also involved in the retinal gliotic response to lesion. After injury, microglia become activated, change their morphology from ramified to amoeboid and accumulate at the lesion site. Microglia proliferate and secrete chemokines and cytokines to attract other immune cells or induce cell proliferation. They are involved in recognition and elimination of apoptotic neurons by phagocytosis or by presentation of antigens to infiltrating T-cells and augmentation of the immune response. On the other hand, they can also down-regulate T-cell responses or trigger a vicious cycle of persistent activation and recruitment of additional inflammatory cells (Dheen *et al.* 2007; Li *et al.* 2015).

Active cross-talk between microglia and Müller cells, mediated by several neurotrophic factors, can directly influence the release of GDNF, leukemia inhibitory factor (LIF), CNTF and basic fibroblast growth factor (bFGF) by Müller cells (Harada *et al.* 2002; Wang *et al.* 2011). On the other hand, activated Müller cells secrete diazepam binding inhibitor (DBI), a ligand for the translocator protein 18 kDa (TSPO), which is expressed in activated microglial cells and limits microglial reactivity during development (Karlstetter *et al.* 2014). The same mechanism applies to Müller cell gliosis during retinal degeneration, when DBI release provides an inhibitory signal to excessive microglial activation (Wang *et al.* 2014). Suppression of microglial immune response has been shown to decrease RGC death and increase axon regeneration in several studies (Heiduschka and Thanos 2006; Sun *et al.* 2013; Cukras *et al.* 2012; Levkovitch-Verbin *et al.* 2014).

3.4 Reactive gliosis as a therapeutic target

Glial neuroprotective and detrimental responses after lesion have been the focus of extensive research, however, the underlying mechanisms and the timing of these responses are not fully understood (Bringmann *et al.* 2009; Johnson and Morrison 2009; Vázquez-Chona *et al.* 2011). Recently, three phases discriminated by gene expression patterns have been described for the gliotic response of macroglia cells after ischemia/reperfusion injuries. In the first phase at reperfusion onset, differentially expressed genes are related to changes in metabolism. In contrast, in the second phase, 24 hours after lesion, enhanced inflammatory and immune responses as well as cell death-related genes become highly regulated. This period is a critical target for the development of interventional therapeutic

approaches. Activation of the complement system pathway characterizes the later stages of the gliotic response (Andreeva *et al.* 2014).

The precise timing of the transition between macroglial supportive and detrimental responses to lesion, and the underlying mechanisms, are poorly understood. The critical role of the glial network in regulating neuronal homeostasis, but also as a detrimental factor after injury or in disease, converts it to a potential therapeutic target for the treatment of neurodegenerative diseases. In order to develop feasible therapeutic approaches, it is critical to determine the mechanisms involved in glial cell activation during injury, including the time course of reactive gliosis and putative detrimental mechanisms.

3.5 Aims of the study

The aims of this study were: 1) to determine the timing of the switch in the Müller cell gliotic response from neuroprotective to detrimental, and 2) to evaluate the effect of an enhanced antioxidative activity in Müller cells on neuronal survival after an acute ischemia/reperfusion injury *in vivo*.

In this study the following hypotheses were tested:

1. Activation of neuroprotective and detrimental gliotic responses in Müller cells after an acute ischemic insult follows a time-dependent pattern.
2. Transient metabolic inhibition of Müller cells during or early after an ischemic lesion leads to increased neuronal degeneration.
3. Transient sub-chronic inhibition of Müller cells leads to increased survival of retinal ganglion cells.
4. Improved neuroprotection after transient inhibition of Müller cell activity is associated with reduced oxidative stress and increased neuronal support.
5. Support of the glial redox homeostasis by overexpression of the mitochondrial enzyme frataxin leads to enhanced neuronal survival.

For this study, an established model of acute glaucoma caused by elevated intraocular pressure (IOP) above systolic pressure was used to induce neuronal degeneration. Increased IOP leading to retinal ischemia is a major risk-factor for open-angle glaucoma (Coleman and Miglior 2008). Hallmarks of the ischemic lesion include energy failure, increased calcium influx followed by neuronal depolarization, excitotoxicity, increased oxidative stress, and withdrawal of neurotrophic factors. Re-oxygenation of the tissue after ischemia promotes neuronal degeneration by the additional production of reactive oxygen

species (ROS), which further potentiate inflammatory responses. Inflammation, which can support tissue repair mechanisms, but can also lead to chronic detrimental effects, further triggers ROS production and tissue damage. This cascade finally results in the death of neurons, glial and endothelial cells.

In the first part of the study, the temporal pattern of the Müller cell gliotic response during and after an ischemic injury was analyzed. For this, the specific glial toxin fluorocitrate (FC) was injected into the eye vitreous at different time points before and after lesion onset in order to transiently inhibit the Müller cell metabolism and activity. FC has already been used to transiently impair glial cell metabolism in the brain (Paulsen *et al.* 1987) and rat retina (Zeevalk and Nicklas 1997). FC impairs glial activity by inhibiting the mitochondrial enzyme aconitase (Cheng *et al.* 1972). Aconitase is a key enzyme of the citric acid cycle (Krebs cycle). FC binds to the aconitase and blocks its activity, thereby, disrupting the Krebs cycle, mainly in glial cells.

In the second part of the study, the effect of frataxin (FXN) overexpression in Müller cells on neuronal survival after an ischemic injury was evaluated. The mitochondrial enzyme FXN is upregulated in several tumor cell lines in response to hypoxic stress (Guccini *et al.* 2011) and is part of the intrinsic retinal response to ischemia (Schultz *et al.* 2016). FXN is involved in iron homeostasis and biogenesis of iron–sulfur clusters, which are part of the active sites of complexes I and II of the mitochondrial electron transport chain and of the enzyme aconitase (Bulteau 2004; Sutak *et al.* 2008). FXN deficiency in mice leads to changes in metabolism by insufficient assembly of iron-sulfur clusters and increased production of harmful free radicals through the Fenton reaction (Al-Mahdawi *et al.* 2006). In contrast, elevated FXN levels reduce the effects of oxidative stress and increase cell survival after oxidative stress-induced cell death (Shoichet *et al.* 2002; Runko *et al.* 2008). To evaluate the effects of increased FXN expression, two mice models overexpressing FXN were generated: one expressing FXN ubiquitously in the retina and one specifically in retinal Müller cells.

4 Summary of the manuscripts

Increased frataxin levels protect retinal ganglion cells after acute ischemia/reperfusion in the mouse retina in vivo, Rowena Schultz, Otto W. Witte, Christian Schmeer, published in the journal **Investigative Ophthalmology and Visual Science**, 2016, volume 57(10), pp.4115–4124.

In this part of the study, it was shown for the first time that the endogenous expression of the mitochondrial enzyme frataxin is upregulated at mRNA and protein levels in response to a transient retinal ischemia *in vivo*. Furthermore, ubiquitous frataxin overexpression significantly increased neuronal survival after lesion. This was associated with an up-regulation of antioxidant enzymes further indicating that frataxin induces neuroprotection by decreasing oxidative stress.

My contribution to this study consisted of the planning and independent realization of the experimental work, including mRNA and protein analysis *via* qPCR and Western blot, microscopic image acquisition, analysis and graphical presentation, data gathering and statistical evaluations, as well as partial preparation of the manuscript. This amounts to 80% of the work.

Transient inhibition of Müller cells by fluorocitrate increases neuronal survival after acute retinal ischemia in vivo, Rowena Schultz, Twinkle Vohra, Julia Lindner, Otto W. Witte, Christian Schmeer, under review at the **Journal of Neurochemistry** (submitted on September 29, 2017)**.**

For this part of the study, Müller cell metabolism was transiently impaired using the gliotoxin fluorocitrate to evaluate the time-dependent activation of the Müller cell gliotic response after acute ischemia/reperfusion injury. Inhibition of the metabolism of Müller cells in the acute phase after ischemia but not before or at lesion onset increased neuronal survival. Furthermore, the expression of antioxidant enzymes and growth factors was increased after diminished Müller cell metabolism, which supports the notion that the gliotic response of Müller cells seem to turn detrimental at later time points after acute ischemia. Therefore, glia cells provide a feasible target to improve neuronal survival after an ischemic injury.

My contribution to this study consisted of the planning and independent realization of the experimental work, in particular the biochemical assays and microscopic image acquisition. This was done in collaboration with the master student Twinkle Vohra. Cell culture experiments were established and performed in collaboration with the master student Julia Linder. In addition, I performed all mRNA analyses via qPCR, data gathering and statistical evaluations, and graphical presentation as well as preparation and revision of the manuscript. This amounts to 70% of the work.

Frataxin overexpression in Müller cells protects retinal ganglion cells in a mouse model of acute glaucoma in vivo, Rowena Schultz, Melanie Krug, Michel Precht, Stefanie G. Wohl, Otto W. Witte, Christian Schmeer, has been submitted to **Scientific Reports** on November 17, 2017.

For this part of the study, frataxin was specifically overexpressed in Müller cells to answer the question whether specific modulation of Müller cells would improve neuroprotection after an acute ischemic injury. Elevated frataxin in Müller cells resulted in increased RGC survival after acute retinal ischemia/reperfusion and altered expression of antioxidant enzymes and growth factors. These findings further support the notion that the targeted modulation of glial cells and their gliotic response is a putative approach to improve neuronal survival after injury in the central nervous system.

My own contribution to this study consisted of the planning and independent realization of all experimental work, establishment of the mRNA and protein analysis *via* qPCR and Western blot, microscopic image acquisition and graphical presentation, data gathering and statistical evaluations, as well as preparation and revision of the manuscript. This amounts to 80% of the work.

Glaucoma

Increased Frataxin Levels Protect Retinal Ganglion Cells After Acute Ischemia/Reperfusion in the Mouse Retina In Vivo

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Purpose. The mitochondrial protein frataxin (FXN) is highly expressed in metabolically active tissues and has been shown to improve cell survival in response to oxidative stress after ischemia. Retinal ischemia/hypoxia is a complication of ocular diseases such as diabetic retinopathy and glaucoma. There are no effective therapeutic approaches currently available. This study was performed to evaluate the neuroprotective effects of FXN after acute retinal ischemia/reperfusion in vivo.

Methods. Retinal ischemia/reperfusion was induced in adult wild-type and FXN-overexpressing mice by transient elevation of intraocular pressure (IOP) for 45 minutes. Expression of FXN was evaluated by quantitative (q)RT-PCR and Western blot analysis between 6 and 48 hours after ischemia. Retinal ganglion cell (RGC) survival was determined with immunofluorescent staining and fluorescence microscopy 14 days after lesion. Expression of hypoxia-inducible factors *Hif-1α* and *Hif-2α* and of oxidative stress markers heme oxygenase-1 (*Hmox1*), glutathione peroxidase 1 (*Gpx1*), superoxidase dismutase 1 and 2 (*Sod1*, *Sod2*), and catalase was evaluated by qRT-PCR.

Results. Endogenous FXN levels were upregulated for up to 24 hours after retinal ischemia in vivo. Retinal ganglion cell survival was significantly improved in FXN-overexpressing mice 14 days after ischemia. Expression of antioxidative enzymes *Gpx1*, *Sod2*, and catalase was significantly increased in FXN-overexpressing mice after lesion.

Conclusions. Retinal FXN levels are increased in response to ischemia. Furthermore, elevated FXN levels had a clear neuroprotective effect as shown by increased ganglion cell survival after acute retinal ischemia/reperfusion. Frataxin's neuroprotective effect was associated with an upregulation of antioxidative enzymes. The data suggest that FXN induces neuroprotection by decreasing oxidative stress.

Keywords: frataxin, retinal ischemia, retinal ganglion cell, antioxidants

Frataxin (FXN) is a nuclear-encoded mitochondrial protein highly conserved among eukaryotes. The mouse gene (*Frda*) encodes a 207–amino acid protein showing 73% amino acid identity to its human counterpart.[1] It is abundantly expressed in metabolically active tissues such as liver, skeletal and cardiac muscle, and brain.[1] In the retina of normal mice, immunoreactivity to FXN is found in the external and internal plexiform layers, the ganglion cell layer, and the inner nuclear layer.[2] Frataxin deficiency is closely related to Friedreich ataxia (FRDA), a neurodegenerative disease associated with abnormal influx of iron into the mitochondria, which increases the susceptibility of the nervous system to oxidative stress.[3–8] Furthermore, complete absence of FXN leads to early embryonic lethality in transgenic mice. Conditional FXN knockout strains for striated muscles and neuron/cardiac muscles reproduce progressive pathophysiological and biochemical features of the human disease and demonstrate time-dependent intramitochondrial iron accumulation.[9] The abnormal use of iron leads to changes in metabolism and increased production of harmful free radicals.[10,11] Although the biological functions of FXN are still not fully understood, the protein serves important functions in iron homeostasis and biogenesis of iron–sulphur clusters in active sites of the complexes I and II of the mitochondrial electron transport chain and aconitase.[12,13] Cells that are deficient in FXN appear to be less efficient at generating natural antioxidant factors.[14] There is evidence indicating that increasing FXN reduces the effects of oxidative stress and increases cell survival after oxidative stress–induced cell death.[3,4,15,16]

Retinal ischemia/hypoxia is a complication of ocular diseases such as diabetic retinopathy, retinopathy of prematurity, and glaucoma.[17] A hallmark of the pathologic alteration in retinal ischemia is the generation of excessive reactive oxygen species (ROS) during reperfusion, which is involved in neuronal cell death.

To date, the effect of ischemia on FXN levels and of FXN overexpression on oxidative stress levels and neuronal survival after acute retinal ischemia/reperfusion has not been evaluated.

The aims of this study were to determine in vivo the effect of a transient ischemia/reperfusion on the expression of retinal FXN, whether increased FXN levels improve retinal ganglion cell (RGC) survival after an ischemic lesion, and the effect of increased FXN on the expression of antioxidative enzymes after ischemia.

Materials and Methods

Animal Guidelines

Male wild-type C57BL/6J and FXN-overexpressing mice, weighing 24.9 ± 2.4 g, aged 3 months, were used. Transgenic animals overexpressing FXN were generated by breeding homozygous mice expressing the *Cre* gene under the transcriptional control of a human cytomegalovirus (CMV) minimal promoter[18] (Deleter, kindly provided by Christian Hübner, Department of Human Genetics, Jena University Hospital, Germany) to heterozygous mice carrying a CMV-driven human FXN cDNA preceded by a loxP-flanked stop cassette (B6 Rosa 26 Frataxin, developed by Michael Ristow, Department of Health Sciences and Technology, Swiss Federal Institute of Technology, Zürich, Switzerland). Cre-mediated excision of the stop cassette leads to expression of human frataxin (hFXN) and of green fluorescent protein (GFP). Hereafter, transgenic mice are referred to as Deleter B6 FXN.

All experiments were performed in accordance with the European Convention for Animal Care and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were also approved by the local Animal Care Committee. Animals were kept in standard cages in groups of five on a 14-hour light/10-hour dark cycle, with food and water available ad libitum, temperature ranging from 22°C to 25°C, and humidity ranging between 55% and 60%. Mice were randomized for each experimental setup into a nonlesioned group (C57BL/6 $n = 28$; Deleter B6 FXN $n = 14$) and an ischemia group (C57BL/6 $n = 55$; Deleter B6 FXN $n = 16$).

Transient Retinal Ischemia

Transient retinal ischemia (TRI) was induced by elevating the intraocular pressure above systolic pressure.[19,20] Briefly, mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate in phosphate-buffered saline (PBS; 500 mg/kg body weight; Fluka, Seelze, Germany). After application of topical anesthesia (oxybuprocaine-hydrochloride 4 mg/mL; Bausch & Lomb GmbH, Feldkirchen, Germany), the anterior chamber of the right eye was cannulated with a 30-gauge needle connected to an elevated normal saline reservoir. Intraocular pressure (IOP) was elevated above systolic pressure for 45 minutes. For sham procedures, a needle attached to a saline reservoir was inserted into the anterior chamber, but pressure was not increased. Intraocular pressure increase and maintenance were evaluated during the procedure using an induction/impact tonometer (TonoLab; Tiolat Ltd., Helsinki, Finland). This method has been validated for the mouse eye.[21] One drop of antibiotic solution (ofloxacin; Bausch & Lomb GmbH, Berlin, Germany) was applied topically to the treated eye after cannulation. After the corresponding period of ischemia, the needle was withdrawn from the anterior chamber, and the IOP was normalized. Treated eyes were covered with eye ointment (Panthenol; Jenapharm, Jena, Germany) and inspected daily. Animals with signs of inflammation or iatrogenic cataract were excluded from further analysis.

In order to determine the appropriate duration of ischemia, we evaluated RGC survival after 30, 45, or 60 minutes of ischemia. An ischemic duration of 30 minutes induced a slight loss of RGCs, whereas 60 minutes of ischemia resulted in less than 15% of RGCs surviving the insult. Therefore, we chose 45 minutes of retinal ischemia to perform all further analyses. Our results are in agreement with previous studies.[19,20]

Immunostaining and Evaluation of RGC Numbers

In order to evaluate RGC survival following ischemia, animals were killed with an overdose of chloral hydrate (30%) 14 days after TRI. For immunostaining, eyes were enucleated and retinae were removed and fixed by immersion in 4% paraformaldehyde for 20 minutes. After washing in PBS, retinal whole mounts were flattened by making incisions from the periphery halfway to the optic nerve to form four symmetric lobes and permeabilized with 0.3% Triton X-100 for 45 minutes at room temperature (RT). Blocking was achieved by incubation with 3% bovine serum albumin (BSA) and 10% normal donkey serum (NDS) in PBS supplemented with 0.3% Triton X-100 for 2 hours at RT. After nonspecific binding was blocked, the tissue was incubated with primary antibody raised against the RGC specific transcription factor Brn3a (1:300; Santa Cruz, Heidelberg, Germany) in 2% NDS overnight at 4°C. After washing with PBS, retinae were incubated with corresponding secondary antibody (Molecular Probes, Leiden, The Netherlands) in 10% NDS for 1 hour at RT. Specificity of the staining was tested by incubation without primary antibody.

Retinal ganglion cell survival was evaluated on retinal whole mounts 14 days post ischemia. Labeled RGCs were counted in single fields at three different retinal eccentricities in each of the four whole-mount lobes (1/6, 3/6, and 5/6 from retinal radius; 0.093 mm^2 each, 12 fields in total) by means of fluorescence microscopy according to Schmeer et al.[22] The number of surviving RGCs was expressed as number of cells per square millimeter, and cell counts were given as percentage of corresponding contralateral and unlesioned eyes.

Quantitative Polymerase Chain Reaction (qRT-PCR) Analysis

Unlesioned and ischemic animals were killed with a chloral hydrate overdose (30%) 6, 12, 24, and 48 hours after lesion. Eyes were enucleated and retinae were shock frozen in liquid nitrogen. Isolation of mRNA was carried out using the RNeasy micro kit (Qiagen, Hilden, Germany), and equal amounts of total retinal mRNA were reverse transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Schwerte, Germany). Quantitative RT-PCR amplification of murine frataxin (*mFxn*), *hFXN*, hypoxia-inducible factors 1 and 2 (*Hif-1α*, *Hif-2α*), glial fibrillary acidic protein (*Gfap*), and the antioxidative stress markers heme oxygenase-1 (*Hmox1*), glutathione peroxidase 1 (*Gpx1*), and superoxide dismutase 1 and 2 (*Sod1* and *Sod2*), as well as catalase (*Cat*), was carried out with Brilliant II SYBR Green QPCR Mastermix (Agilent Technologies, Santa Clara, CA, USA) using the primer sequences depicted in the Table. After denaturizing at 95°C for 10 minutes, 40 amplification cycles were carried out as follows: denaturation at 95°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds. The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine–guanine phosphoribosyltransferase (*Hprt*) were used as reference genes. To determine the relative expression ratio (fold change) of the target genes, the analysis introduced by Pfaffl et al.[23] was applied.

Western Blotting

In order to evaluate changes in the protein levels of retinal FXN in Deleter B6 FXN mice and C57BL/6 mice following increased IOP, we performed Western blotting. Animals were killed with an overdose of chloral hydrate (30%) 12 and 48 hours after

Table. Quantitative RT-PCR Primer Sequences for mRNAs Analyzed in the Study, Together With Their Expected Product Length, GenBank Accession Number, and Reference

mRNA		Primer Sequence	Product Size	Accession No.	Reference
hFXN	fw	5′ CCTTGCAGACAAGCCATACA 3′	150 bp	NM_000144.4	41
	re	5′ CCACTGGATGGAGAAGATAG 3′			
mFxn	fw	5′ CCTGGCCGAGTTCTTTGAAG 3′	152 bp	U95736.1	42
	re	5′ GCCAGATTTGCTTGTTTGG 3′			
Gpx1	fw	5′ GGGACTACACCGAGATGAACGA 3′	197 bp	NM_008160	43
	re	5′ ACCATTCACTTCGCACTTCTCA 3′			
Sod1	fw	5′ GTCCGTCGGCTTCTCGTCT 3′	163 bp	NM_011434.1	44
	re	5′ CACAACTGGTTCACCGCTTG 3′			
Sod2	fw	5′ ATTAACGCGCAGATCATGCA 3′	161 bp	NM_013671.3	43
	re	5′ TGTCCCCCACCATTGAACTT 3′			
Cat	fw	5′ GCAGATACCTGTGAACTGTC 3′	229 bp	NM_009804.2	45
	re	5′ GTAGAATGTCCGCACCTGAG 3′			
Hmox1	fw	5′ GGTGATGGCTTCCTTGTACC 3′	155 bp	NM_010442.2	46
	re	5′ AGTGAGGCCCATACCAGAAG 3′			
Hif-1α	fw	5′ CAACGTGGAAGGTGCTTCA 3′	242 bp	NM_010431.2	
	re	5′ CGGCTCATAACCCATCAACT 3′			
Hif-2α	fw	5′ AGGTCTGCAAAGGACTTCGG 3′	162 bp	NM_010137.3	
	re	5′ CAAGTGTGAACTGCTGGTGC 3′			
Gfap	fw	5′ AGAAAGGTTGAATCGCTGGA 3′	176 bp	NM 010277.3	47
	re	5′ GCCACTGCCTCGTATTGAGT 3′			
Hprt	fw	5′ TGACACTGGTAAAACAATGCA 3′	94 bp	NM_013556.2	48
	re	5′ GGTCCTTTTCACCAGCAAGCT 3′			
Gapdh	fw	5′ AGGTCGGTGTGAACGGATTTG 3′	123 bp	NM_001289726.1	49
	re	5′ TGTAGACCATGTAGTTGAGGTCA 3′			

fw, forward primer; re, reverse primer.

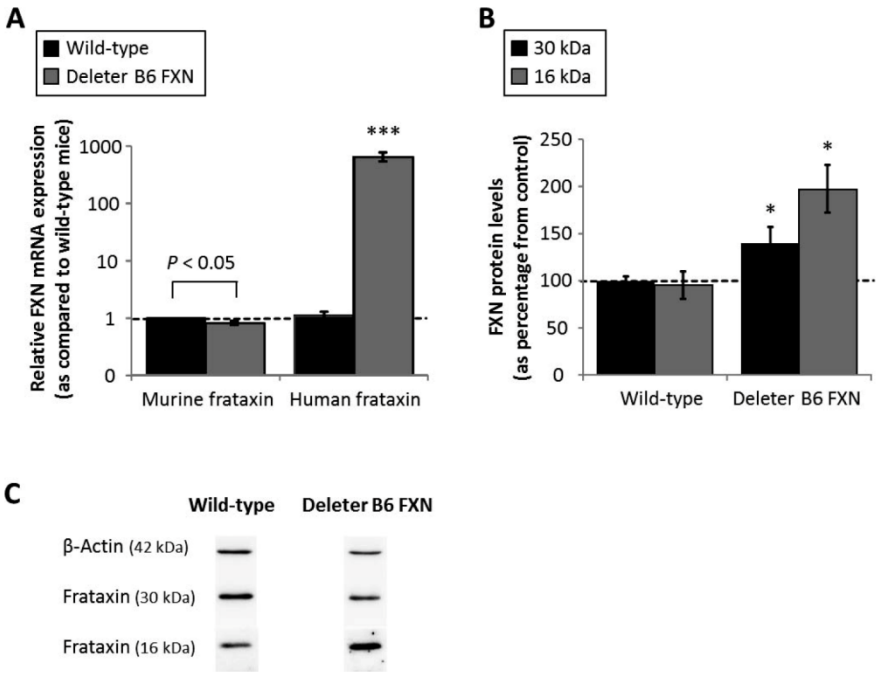

Figure 1. Overexpression of human frataxin in the retina from Deleter B6 FXN mice. (**A**) Relative expression of human frataxin (hFXN) mRNA was determined by quantitative polymerase chain reaction, normalized against *Gapdh* and *Hprt* mRNA levels and reported as fold increase of expression in wild-type C57BL/6 mice. The *y*-axis is depicted in logarithmic scaling. *Bars* represent the mean ± SEM; *dashed line* represents 1.0 ratio, $^{***}P < 0.001$ versus naïve C57BL/6 control; $n = 6$. (**B**) Frataxin protein levels of precursor frataxin form (30 kDa) and mature frataxin form (16 kDa) were determined by densitometric analysis of Western blotting in wild-type C57BL/6 and Deleter B6 FXN mice. Values are expressed as percentage of naïve C57BL/6 mice and normalized against β-actin. *Bars* represent mean ± SEM; *dashed line* represents 100%, $n = 4$, $^{*}P < 0.05$ versus C57BL/6. (**C**) Representative Western blots showing precursor and mature bands for frataxin in wild-type and Deleter B6 FXN naïve mice.

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lesion and eyes were enucleated. Retinae were dissected, harvested in RIPA lysis buffer (Santa Cruz), homogenized, and then centrifuged at 10,000*g* for 10 minutes at 4°C. Protein concentration was determined using the Quickstart Bradford Protein Assay Kit 3 (BioRad, München, Germany). Proteins (10–20 μg) were loaded onto an SDS 12% polyacrylamide gel. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Wuppertal, Germany). Nonspecific binding was blocked with 5% skim milk in Tween/Tris-buffered saline (TBS-T). Membranes were incubated overnight at 4°C with primary antibodies raised against FXN (1:500; Santa Cruz) and β-actin (1:2000; Abcam, Cambridge, UK) prepared in 2% skim milk in TBS-T. Membranes were washed with TBS-T and incubated with a 1:5000 dilution of horseradish peroxidase (HRP)–conjugated secondary antibody for 60 minutes. Protein bands were visualized using an enhanced chemiluminescence reaction kit (Immun-Star WesternC Chemiluminescence Kit, Bio-Rad), photographed with Fujifilm LAS-3000 Imager (Fuji Photo Film, Düsseldorf, Germany), and analyzed with ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij; Version 1.46). Measurements were done in quadruplicate. Each band was normalized against the corresponding β-actin band. Changes in protein expression were expressed as percentage from unlesioned control levels.

Statistical Analysis

All results are expressed as the mean ± standard error of the mean (SEM). Each group consisted of at least five animals. For RGC survival analysis, statistical significance was assessed using 1-way ANOVA followed by Holm-Sidak post hoc analysis by comparing wild-type control mice and treatment groups. For relative gene expression values (ratio), 1-way ANOVA followed by Holm-Sidak post hoc analysis was applied between treatment groups and control. Two-way ANOVA followed by Holm-Sidak post hoc analysis was applied for comparison of lesion effects between mice strains. For Western blot analysis, results were compared by 2-way ANOVA followed by Holm-Sidak post hoc analysis comparing wild-type and transgenic control mice and ischemic groups. SigmaPlot version 13.0 (Systat, Erkrath, Germany) was used, and the level of significance was set at $P < 0.05$.

Results

Frataxin mRNA and Protein Levels Are Increased in the Retina of FXN-Overexpressing Mice

In Deleter B6 FXN mice, differentiation between mFXN and hFXN was possible only at the mRNA level. Primers were specifically designed for the sequence corresponding to the N-terminal region of the protein, which shows the highest diversity between species, whereas the antibody for Western blotting was directed against the C-terminal mitochondrial sequence, which is highly conserved between eukaryotes.

Human FXN expression was 650 times higher than endogenous mouse FXN ($P < 0.001$). Importantly, in the presence of hFXN, mRNA expression of endogenous mouse *Fxn* was decreased by 20% (1.00 ± 0.01 fold change versus 0.81 ± 0.05 fold change, $P < 0.05$) (Fig. 1A). In order to evaluate changes at the protein level, we measured levels of FXN precursor and mature forms (30 and 16 kDa, respectively). Relative levels of the 30-kDa form were 39 ± 12% higher compared to those in wild-type mice ($P < 0.05$), whereas the 16-kDa form was increased by 97 ± 20% ($P < 0.05$) (Fig. 1B).

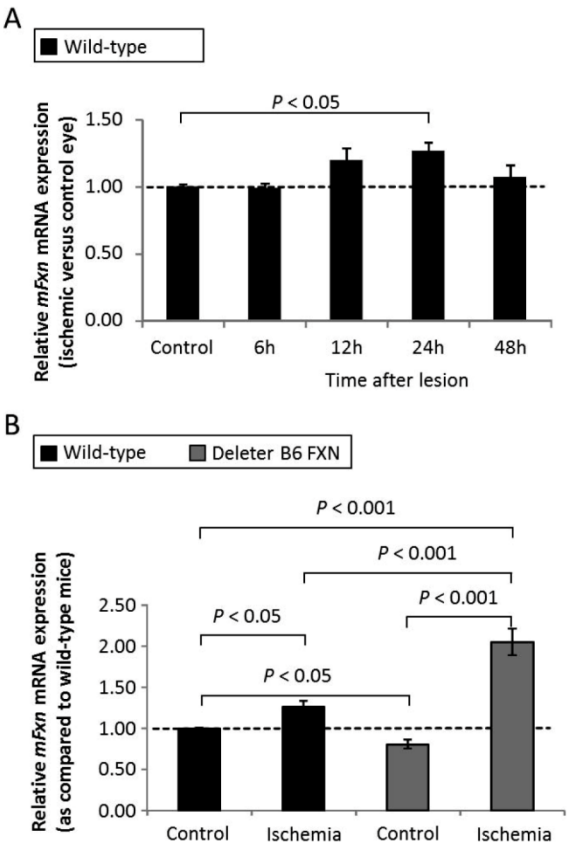

Figure 2. Effect of acute retinal ischemia/reperfusion on mRNA levels of frataxin, depicted as the ratio of treated versus naïve control eyes. Frataxin mRNA was determined by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt* mRNA levels. (**A**) Relative expression of murine frataxin in wild-type mice at different times of reperfusion. (**B**) Relative expression of murine frataxin in wild-type and Deleter B6 FXN mice as compared to naïve wild-type, after 24 hours of reperfusion. *Bars* represent the mean ± SEM; *dashed lines* represent 1.0 ratio, $n = 5$ to 6. *P* values as depicted versus unlesioned wild-type C57BL/6 control.

Levels of FXN mRNA and Protein Are Increased in the Retina After Transient Ischemia/Reperfusion

Frataxin mRNA levels in wild-type mice were significantly increased by 26% after lesion, reaching a peak 24 hours after ischemia (1.26 ± 0.07-fold increase, $P < 0.05$), and decreased again to basal levels after 48 hours of reperfusion (Fig. 2A). In transgenic mice, *mFxn* expression showed a 2-fold increase compared to wild-type mice (2.05 ± 0.16 vs. 1.26 ± 0.07 fold change, $P < 0.001$) (Fig. 2B) after ischemia. Human *FXN* expression did not change after lesion (data not shown).

In order to evaluate the effect of ischemia on FXN levels, we determined protein content of the precursor and mature forms of FXN by means of Western blotting.

As shown in Figure 3A, the precursor form was not increased in wild-type mice for up to 48 hours after lesion compared to unlesioned control levels (100 ± 10% vs. 102 ± 11% and 112 ± 17%, NS). Protein levels for the mature form of FXN significantly increased 12 hours after lesion (100 ± 8% vs. 208 ± 27% $P < 0.01$) (Fig. 3B) and remained elevated for up to 48 hours after lesion (100 ± 8% vs. 216 ± 30% $P < 0.01$). In

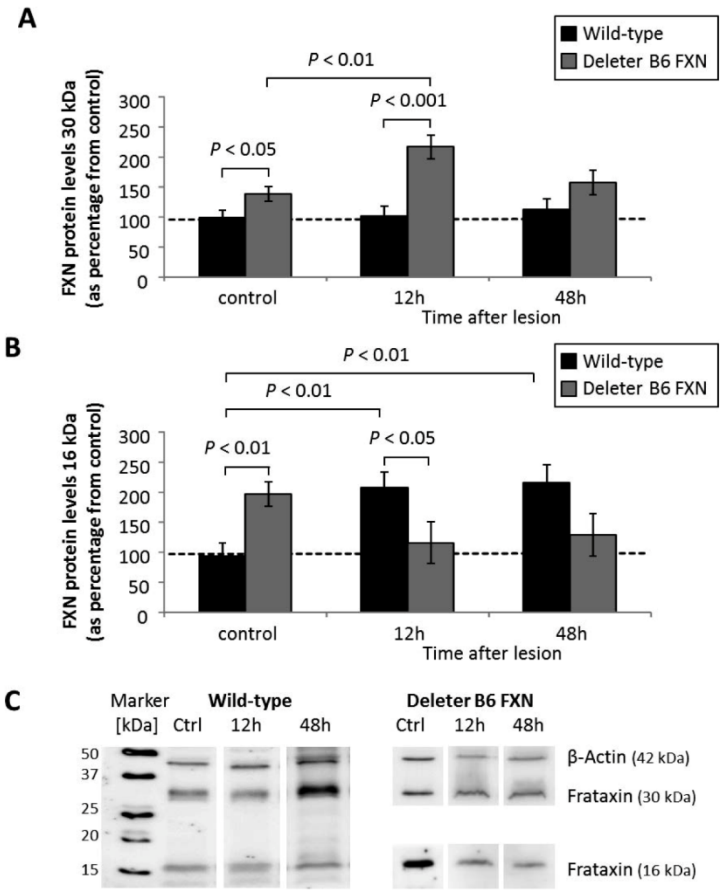

Figure 3. Effect of acute ischemia/reperfusion on protein levels of frataxin 12 and 48 hours after lesion. Densitometric analysis of (**A**) precursor frataxin (30 kDa) and (**B**) mature frataxin (16 kDa) protein levels by Western blotting in naïve and ischemic retinae. Values are expressed as percentage from unlesioned wild-type control retinae (100%). *Bars* represent mean ± SEM; *dashed line* represents 100%, $n = 5$, P values as depicted versus unlesioned wild-type C57BL/6 control. (**C**) Representative Western blots showing precursor and mature bands for frataxin.

transgenic mice, levels of the FXN precursor form were increased 12 hours after lesion compared to those in unlesioned animals (139 ± 12% vs. 217 ± 20%, $P < 0.01$, Fig. 3A), whereas the mature form of FXN did not change (197 ± 20% vs. 117 ± 35%, NS, Fig. 3B). Furthermore, levels of the mature form of FXN were decreased compared to those in wild-type mice 12 hours (208 ± 27% vs. 117 ± 35%, $P < 0.05$) after ischemia (Fig. 3B).

Retinal Ganglion Cell Survival Is Significantly Increased in FXN-Overexpressing Mice After Acute Ischemia/Reperfusion

Retinal ganglion cell density was analyzed in wild-type and transgenic mice at different retinal eccentricities (1/6, 3/6, and 5/6 from retinal radius). Values for the number of cells were normalized against the corresponding contralateral eye. Ganglion cell numbers were indistinguishable between unlesioned wild-type and transgenic mice ($P > 0.05$, data not shown). Therefore, contralateral eyes from each strain were used to normalize for the number of surviving RGCs after lesion.

Fourteen days after lesion, the percentage of surviving RGCs was significantly reduced both in wild-type (32.8 ± 5.2%; $n = 6$) and in transgenic mice (65.2 ± 4.5%, $n = 5$) compared to the corresponding unlesioned control animals ($P < 0.05$). However, FXN-overexpressing mice had a 32.4% higher number of RGCs surviving after lesion compared to wild-type mice ($P < 0.001$, Fig. 4).

Expression of Hypoxia-Inducible Factor and Antioxidative Markers Is Increased in FXN-Overexpressing Mice After Lesion

To determine whether increased antioxidative capacity might be involved in FXN-mediated neuroprotection, we evaluated the gene expression of antioxidative enzymes *Hmox1*, *Gpx1*, *Sod1* and *2*, and *Cat* by means of qRT-PCR. Expression analysis was performed at 24 hours of reperfusion to match FXN peak expression induced by the ischemic lesion.

In wild-type mice, expression levels of *Hmox1*, *Gpx1*, and *Sod1* were significantly increased after lesion (Ho-1: 9.21 ± 1.74-fold increase, $P < 0.001$, Fig. 5A; *Gpx1*: 1.33 ± 0.06-fold increase, $P < 0.001$, Fig. 5B; *Sod1*: 1.12 ± 0.06-fold increase, $P < 0.05$, Fig. 5D), whereas *Cat* expression was decreased (0.74

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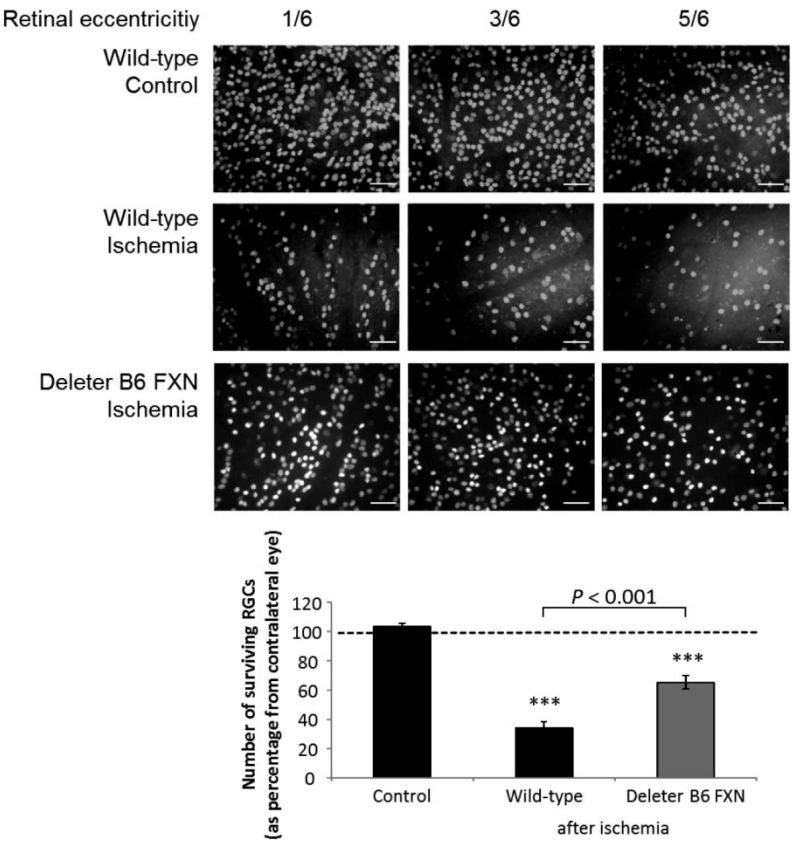

Figure 4. Effect of frataxin overexpression on RGC survival 14 days after acute retinal ischemia/reperfusion. (**A**) Images of retinal whole-mount preparations from lesioned and unlesioned wild-type and Deleter B6 FXN animals at various retinal eccentricities (1/6, 3/6, 5/6 from retinal radius) are shown. Images were taken with a fluorescent microscope (×40). *Scale bars* represent 50 μm. (**B**) Analysis of surviving RGCs after acute retinal ischemia. Numbers of RGCs are expressed as percentages from contralateral eyes. Cell counts were determined 14 days after the insult. *Bars* represent the mean ± SEM; *dashed line* represents 100%, $n = 5$–9. $^{***}P < 0.001$ versus unlesioned C57BL/6 control.

± 0.02-fold decrease, $P < 0.001$, Fig. 5C) compared to unlesioned controls.

In Deleter B6 FXN mice, expression of *Hmox1* (4.45 ± 1.53), *Gpx1* (1.66 ± 0.1), *Sod1* (1.25 ± 0.05), Sod2 (1.45 ± 0.1), and *Cat* (1.39 ± 0.04) was significantly higher compared to the corresponding unlesioned control 24 hours after lesion ($P < 0.001$) (Figs. 5A–E).

Gene expression 24 hours after lesion changed significantly between wild-type and Deleter B6 FXN mice for *Gpx1* (1.33 ± 0.06 vs. 1.66 ± 0.1-fold increase, $P < 0.001$), *Sod2* (1.13 ± 0.02 vs. 1.45 ± 0.1-fold increase, $P < 0.001$), *Hmox1* (9.21 ± 1.74 vs. 4.45 ± 1.53-fold decrease, $P < 0.05$), and *Cat* (0.74 ± 0.02 vs. 1.39 ± 0.04-fold increase, $P < 0.001$) (Figs. 5A–E).

Expression of *Gfap* significantly increased after lesion in wild-type and Deleter B6 FXN mice (5.85 ± 0.27 and 4.51 ± 0.28, $P < 0.001$). Although the *Gfap* increase in transgenic mice was slightly lower compared to wild-type, this was not statistically significant ($P = 0.081$, Fig. 5F).

Expression levels of both *Hif-1α* and *Hif-2α* were not changed after lesion in C57BL/6 wild-type mice but significantly increased in Deleter B6 FXN (*Hif-1α* 1.08 ± 0.04 vs. 1.54 ± 0.04 and *Hif-2α* 0.92 ± 0.03 vs. 1.37 ± 0.05 fold change, $P < 0.001$) (Figs. 6A, 6B).

Discussion

Summary of Results

In the present work, we found an upregulation of the mitochondrial enzyme FXN both at the mRNA and at the protein level after retinal ischemia, indicating its involvement in the response to lesion in the adult mouse eye in vivo. Importantly, we found increased RGC survival after ischemia in transgenic mice overexpressing FXN. Improved cell survival correlated with an increase in gene expression of antioxidative-related enzymes.

Frataxin Overexpression Increases RGC Survival After Acute Ischemia/Reperfusion In Vivo

Here, we show that FXN levels are upregulated after acute retinal ischemia/reperfusion with a peak at 24 hours of reperfusion. To our knowledge, this is the first study showing an involvement of FXN in the response to ischemia/reperfusion in the central nervous system in vivo. Our findings are in agreement with other studies reporting FXN upregulation in response to hypoxic stress in vitro.[24,25] Upregulated FXN expression is also found in several tumor cell lines in response to hypoxic stress and promotes tumor

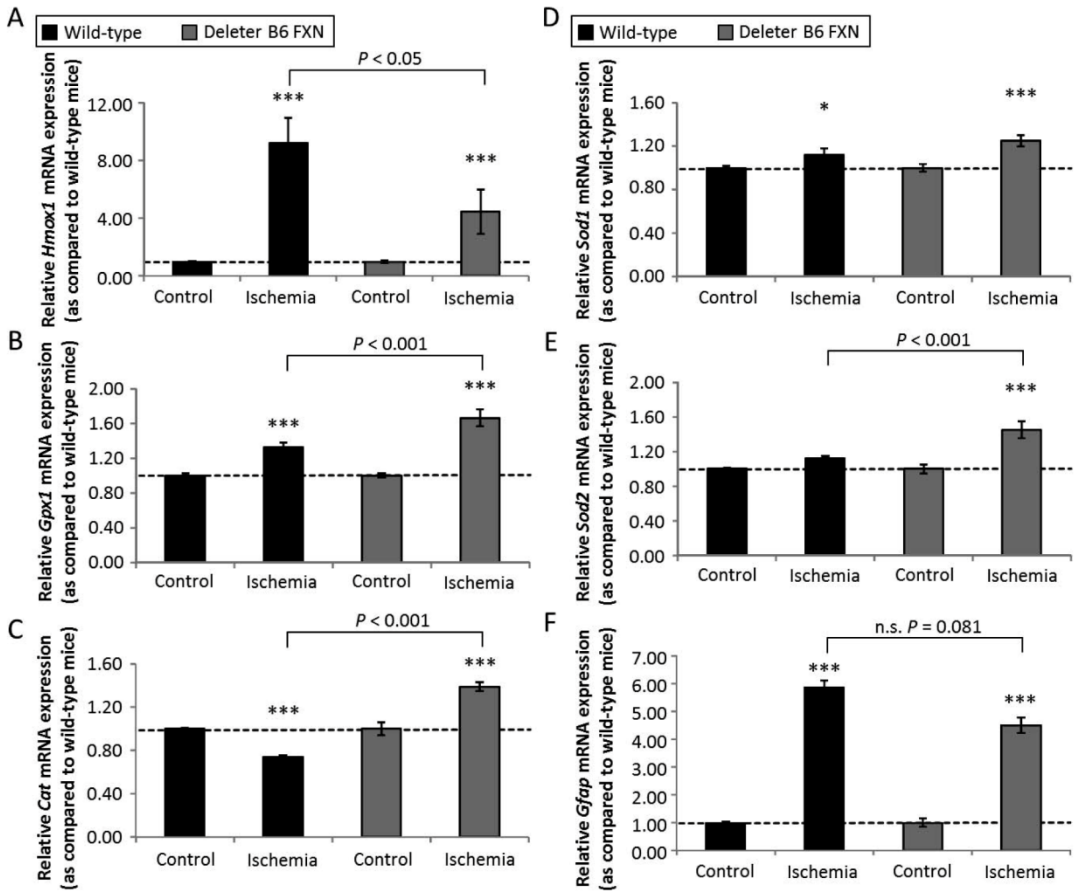

Figure 5. Effect of acute retinal ischemia/reperfusion on mRNA levels of (**A**) *Hmox1*, (**B**) *Gpx1*, (**C**) *Cat*, (**D**) *Sod1*, (**E**) *Sod2*, and (**F**) *Gfap*, depicted as the ratio of treated versus naïve corresponding control eyes. mRNA levels were determined 24 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt* mRNA levels. *Bars* represent the mean ± SEM; *dashed lines* represent 1.0 ratio, $n = 5$ to 6, $^{***}P < 0.001$ versus corresponding unlesioned control.

cell survival and progression.[25] The role of increased FXN levels following retinal ischemia is not clear, but could be part of a response mechanism aimed to overcome lesion-induced iron dysregulation and increased oxidative stress. Recently, an increase in FXN levels was found to have a cardioprotective effect against ischemia/reperfusion.[26] In order to evaluate the effect of increased FXN on neuronal survival following acute retinal ischemia/reperfusion in vivo, we used a conditional mouse model overexpressing FXN under the control of a ubiquitous active promoter. Mice constitutively expressing the full-length hFXN cDNA have been already described and do not show any signs of ataxia or other obvious abnormalities.[27] High levels of FXN were revealed in all major organs affected in FRDA, including the pancreas, brain, skeletal muscle, and the heart.[28] In our mouse model, we found that prelesion overexpression of hFXN and not lesion-induced expression leads to enhanced neuronal cell survival in the retina after acute ischemia/reperfusion in vivo. Our results are in support of other studies showing that FXN delivered prelesion is neuroprotective in different brain regions.[3,16] Frataxin-mediated neuroprotection might involve a preconditioning effect and an induction of long-lasting retinal ischemic tolerance, as has been shown after treatment with the iron chelator deferroxamine.[29] As already mentioned, FXN plays an important role in mitochondrial iron homeostasis.[12,13] In wild-type mice, we found increased levels of FXN mature protein after lesion, whereas the precursor form was not elevated. In contrast, although levels of the precursor protein were significantly increased in transgenic as compared to wild-type mice, the mature protein was not changed after lesion. This might suggest that in wild-type mice FXN is converted into mature protein after lesion, whereas in transgenic mice maximum levels of the mature protein have already been reached before lesion. Therefore, no further increase takes place after lesion, and the precursor form accumulates in the cell. The reason for the lack of increase of FXN's mature form after ischemia in transgenic animals, compared to wild-type, is not clear and needs to be further evaluated. Importantly, translation of FXN mRNA into protein as well as enzyme maturation has been shown to be tightly regulated.[30]

Frataxin Overexpression Leads to an Increased Antioxidative Response

Since oxidative stress is critically involved in cell damage after retinal ischemia, we evaluated the expression levels of

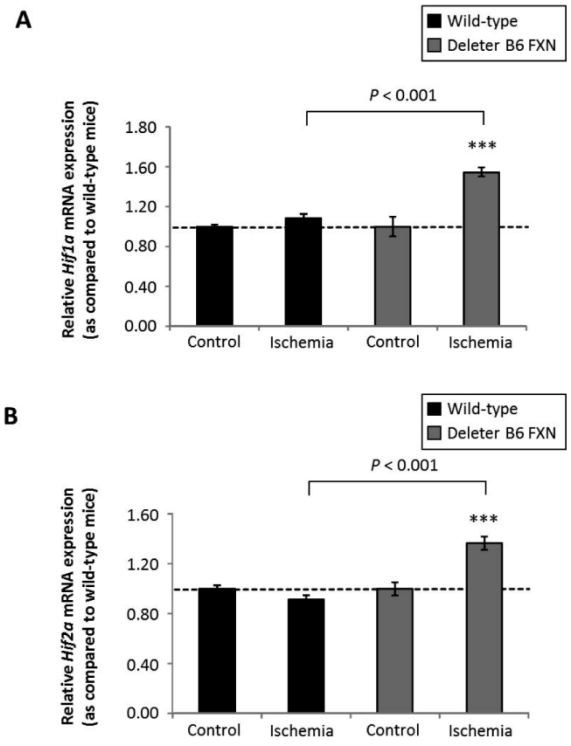

Figure 6. Effect of acute retinal ischemia/reperfusion on mRNA levels of (**A**) *Hif-1α* and (**B**) *Hif-2α*, depicted as the ratio of treated versus naïve corresponding control eyes. mRNA levels were determined 24 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt* mRNA levels. *Bars* represent the mean ± SEM; *dashed lines* represent 1.0 ratio, $n = 5$ to 6, $^{***}P < 0.001$ versus corresponding unlesioned control.

antioxidant enzymes including *Gpx1*, *Sod1*, *Sod2*, *Cat*, and *Hmox1*, all known to be involved in the response to ischemia. It is known that protective effects mediated by FXN overexpression involve several antioxidant mechanisms and activation of mitochondrial energy metabolism.[15,27,28,31] Moreover, Shoichet et al.[15] showed that FXN itself lacks classical antioxidant properties similar to Gpx, Sod, and Cat. In particular, FXN overexpression enhances Gpx1 activity, increases cellular glutathione content, and upregulates Cat, at least in vitro.[4,15,28,32] In our study, following lesion, expression of *Gpx1*, *Sod2*, and *Cat* was significantly enhanced in mice overexpressing FXN compared to wild-type mice. Interestingly, Cat, which is a potent scavenger of H_2O_2 and provides a powerful antioxidant defense in the retina, was found to be reduced after ischemia in wild-type animals, in agreement with previous studies in rats.[33] Increase in Sod2 and Cat has been previously shown to be neuroprotective in the retina; in particular, intravitreal injection of AAV-Sod2 and AAV-Cat protected RGCs and inner neurons from ischemia/reperfusion injury.[34,35] Glutathione peroxidase 1 has been shown to inhibit cell death and, interestingly, glial activation following experimental stroke in mice.[36]

Expression of *Hmox1* was increased 24 hours after lesion, both in wild-type and in FXN-overexpressing mice; however, it was significantly lower in transgenic mice compared to wild-type mice. The reason for this lower *Hmox1* increase in transgenic mice is not clear. Heme oxygenase-1, which has been shown to protect RGCs after ischemia,[37] is known to be induced in Müller cells by oxidative stress 6 hours after reperfusion and peaks at 12 to 24 hours after lesion.[38] The lower *Hmox1* expression might reflect changes in glial activity in transgenic mice; however, since expression analysis was performed in whole retinal lysates, it is not possible to draw conclusions about cell-specific responses. The trend to a lower *Gfap* expression in transgenic animals, although not significant, could also be indicative of changes in glial response due to FXN overexpression.

Therefore, increased expression of antioxidative markers seems to be involved in the improved survival of RGCs after retinal ischemia/reperfusion found in our study. A possible role of glia cells in mediating neuroprotective effects, as suggested by the regulation of some of the markers evaluated, will be further addressed in a future study.

Levels of Hypoxia-Inducible Factors Hif-1α and Hif-2α Are Elevated in the Retinae of FXN-Overexpressing Mice After Ischemia

Hypoxia-inducible factors (HIFs) are the principal regulators of the transcriptional response to hypoxia. These factors regulate the expression of genes containing the conserved hypoxia-responsive element (HRE). In particular, mouse FXN promoter possesses an HRE and its expression is controlled by *Hif-2α*.[24] Furthermore, Sod2 is also regulated by *Hif-2α*.[39] We measured expression levels of the hypoxia-inducible factors *Hif-1α* and *Hif-2α* in both unlesioned retinae and 24 hours after ischemia. We found that expression levels for both factors were similarly increased in FXN-overexpressing mice, but not in wild-type mice at this time point. Mowat et al.[40] found that in the retina, HIFs show an expression peak already 2 hours after ischemia and reach basal levels 24 hours after lesion. Increased expression of HIFs in transgenic mice 24 hours after lesion further supports our assumption that FXN-mediated neuroprotection is due to a preconditioning-induced ischemic tolerance. Interestingly, in the mouse retina, *Hif-2α* expression has been reported to be limited to Müller cells and that of *Hif-1α* to neurons.[40]

Taken together, our findings show that increased FXN expression is part of the cellular response mechanism to ischemia in the retina. The role of the FXN increase after lesion in the normal retina is still not clear, but might constitute an attempt to overcome damage caused by iron dysregulation and increased oxidative stress after retinal ischemia/reperfusion. Reduced levels of the active form of FXN in transgenic mice after ischemia suggest that the protective effects observed are due to a preconditioning mechanism, and not to a FXN-mediated postlesional effect. The increased neuronal survival elicited by FXN overexpression involves an upregulated antioxidative mechanism after acute retinal ischemia/reperfusion in vivo.

Treatments aiming to modulate FXN levels might constitute therapeutic targets to induce ischemic tolerance and reduce neuronal damage caused by mitochondrial dysfunction after ischemia/reperfusion in the retina.

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4.2 Manuscript II

Journal: Journal of Neurochemistry

Title: Transient inhibition of Müller cells by fluorocitrate increases neuronal survival after acute retinal ischemia *in vivo*

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Running title: Müller cell inhibition is neuroprotective

Key words: Müller cell, neuroprotection, retinal ischemia, inflammation, growth factor, oxidative stress

List of abbreviations: *Bdnf*, brain-derived neurotrophic factor; *Cat*, catalase; *Cd68*, cluster of differentiation 68; *Cntf*, ciliary neurotrophic factor; FC, fluorocitrate; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Gdnf*, glial cell line-derived neurotrophic factor; *Gfap*, glial fibrillary acidic protein; *Glast*, glutamate aspartate transporter; *Glul*, glutamine synthetase; *Gpx1*, glutathione peroxidase 1; *Hmox1*, heme oxygenase-1; *Hprt*, hypoxanthine–guanine phosphoribosyltransferase; *Il-1β*, interleukin 1 beta; IOP, intraocular pressure; *Ngf*, nerve growth factor; PBS, phosphate-buffered saline; RGCs, Retinal ganglion cells; *Sod1* and *Sod2*, superoxide dismutase 1 and 2; *Tnf-α*, tumor necrosis factor alpha; TRI, transient retinal ischemia; *Vim*, vimentin;

Abstract

Müller cells are the main glial cell type in the vertebrate retina. After injury, the reactive Müller cells can be neuroprotective or detrimental to neuronal survival. The time point at which the switch from a neuroprotective effect to a detrimental effect occurs has not been properly determined. Furthermore, the impact of transient functional inhibition of Müller cells on the detrimental effects of reactive gliosis after an ischemic lesion has not been investigated.

Therefore, in this study, we evaluated the time-dependent effects of transient Müller cell inhibition on neuronal survival after acute ischemia/reperfusion in the murine eye. Retinal ganglion cell (RGC) death was induced by elevating the intraocular pressure above systolic pressure. Müller cell metabolism was blocked by intravitreal delivery of the gliotoxin fluorocitrate during lesion onset and in the reperfusion phase. RGC survival was analyzed seven days post lesion onset, and the mRNA expression levels of markers for gliosis and retinal homeostasis were evaluated 12, 24 and 48 hours after the ischemic event.

Transient Müller cell inhibition during ischemia did not influence RGC survival; however, metabolic blocking after the onset of reperfusion significantly increased RGC survival. This was associated with decreased expression of gliosis markers and increased levels of antioxidant enzymes, neurotrophic factors and microglial reactivity markers. Thus, the inhibition of Müller cell metabolism after retinal ischemia induction blocked the detrimental effects of reactive gliosis. Our study indicates that selective regulation of Müller cell activity is a feasible approach to diminishing neuronal damage after development of an ischemic lesion.

Introduction

Retinal ischemia is a clinical condition caused by insufficient blood supply to the retina and is a major cause of blindness worldwide. It is associated with various disorders such as diabetic retinopathy, glaucoma, optic neuropathies, stroke, and other retinopathies. The pathological mechanisms of retinal ischemia are still not fully understood, and treatments for this disorder have limited effectiveness (Minhas *et al.* 2012). At the cellular level, ischemic retinal injury consists of a self-reinforcing degenerative cascade involving neuronal depolarization, calcium influx and oxidative stress initiated by energy failure and glutamatergic stimulation (Casson *et al.* 2004). Another pathological mechanism involves

withdrawal of trophic factors and this mechanism has also been suggested to play an important role in the pathogenesis of glaucoma (Almasieh *et al.* 2012).

Müller cell and microglial activation (reactive gliosis) is an early response that is common to virtually every pathological alteration in the retina (Langmann 2007; Bringmann *et al.* 2006). Müller cells are the most abundant glial cell population in the retina and are in contact with all of the other cell types. After lesioning, they protect neurons via the release of neurotrophic factors and free radical scavengers, the uptake of glutamate, and the facilitation of neovascularization (Bringmann *et al.* 2006). However, gliotic alterations of both Müller cells and microglia also lead to the formation of glial scars and the induction of chronic inflammation, which may contribute to neuronal degeneration and edema development in the diseased retina (Bringmann *et al.* 2006; Bringmann et al. 2004; Bringmann *et al.* 2009; Karlstetter *et al.* 2010). Mechanisms involved in the neuroprotective and detrimental responses of Müller cells, as well as their temporal activation pattern following lesion development, are incompletely understood (Johnson and Morrison 2009; Bringmann *et al.* 2009). This dual effect of Müller cell activation is highly relevant to the development of effective therapeutic approaches aimed at reducing the detrimental gliotic effects and improving neuroprotection. Most experimental therapies to reduce ischemic damage are based on prophylactic rather than therapeutic interventions, limiting their clinical relevance. The available evidence indicates that focusing on single targets and mechanisms to induce neuroprotection after ischemia does not work since multiple pathways in diverse cell types are involved (Bringmann *et al.* 2009; Vecino *et al.* 2015).

The aim of this study was to evaluate the temporal pattern of Müller cell activation and the effect of transient inhibition of Müller cell metabolism on murine retinal ganglion cell survival after acute ischemia/reperfusion *in vivo*. Retinal ischemia was induced by acute elevation of the intraocular pressure (IOP) above systolic pressure (Krempler *et al.* 2011; Schultz *et al.* 2016). Transient Müller cell inhibition was induced by intraocular delivery of the gliotoxin fluorocitrate (FC), which is a specific inhibitor of glial metabolism (Virgili *et al.* 1991; Zeevalk and Nicklas 1997). Changes in the expression levels of markers for gliosis, antioxidant enzymes, microglial reactivity, and neurotrophic factors were assessed by qRT-PCR analysis after Müller cell inhibition.

Material and Methods

Materials

D,L-fluorocitric acid barium salt and the Cell proliferation Kit I (MTT) were obtained from Sigma-Aldrich (Munich, Germany). The glutamine assay kit was ordered from Abnova (Taipei City, Taiwan). Oxybuprocaine hydrochloride (4 g/mL) and Ofloxacin are from Bausch & Lomb GmbH (Feldkirchen, Germany). The reagents for the Bradford assay were purchased from BioRad (Munich, Germany). Chloral hydrate was obtained at Fluka (Seelze, Germany). For qPCR analysis the following kits were used: RNeasy micro kit (Qiagen, Hilden, Germany), Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Schwerte, Germany), Brilliant II SYBR Green QPCR MasterMix (Agilent Technologies, Santa Clara, CA, USA). All other chemicals used are from Merck Millipore (Darmstadt, Germany), unless stated otherwise. The following antibodies were used: goat anti-BRN3a (1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA, RRID:AB_2167511) and donkey anti-goat (Alexa Fluor-488 conjugated, Molecular Probes, Leiden, The Netherlands, RRID:AB_142672).

Animal guidelines

Female C57BL6/J mice each weighing 18 to 20 g (12 weeks old), were used. Animals were bred and held at the research facility. All experiments were performed in accordance with the European Convention for Animal Care and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were also approved by the local animal care committee (reference numbers: 02-047/08 and 02-013/11). Animals were housed in standard cages in groups of 5 animals on a 14-hour light/10-hour dark cycle, with food and water available ad libitum, temperature ranging from 22°C to 25°C and the humidity ranging from 55% to 60%.

Fluorocitrate preparation and treatment

For each experiment, FC was freshly prepared according to Paulsen *et al.*, (1987). Briefly, 2.63 mg of D,L-fluorocitric acid barium salt was dissolved in 328.7 µl of 0.1 M HCl. Ba^{2+} was precipitated by adding 13.15 µl of 0.1 M Na_2SO_4. After adding 328.2 µl of 0.2 M Na_2HPO_4, the solution was centrifuged at 1000 x g for 5 minutes, and the pellet was discarded. The supernatant was adjusted to pH 7.4 by adding 6-8 µl of 1 M NaOH and then adjusted with 0.9% NaCl to a final volume of 1 ml (final concentration 3.2 mM).

For *in vivo* studies, FC was delivered via intraocular injection according to standard protocols (Pearce *et al.* 2015; Stewart 2015). Briefly, 0.5 µl saline (0.9% NaCl, vehicle

solution) or 0.5 µl of 3.2 mM FC (final concentration in the eye 0.32 mM) was injected into the vitreous chamber using a 34G microsyringe (5 µl, Hamilton Robotics, Bonaduz, GR, Switzerland). Animals were anesthetized with a gas mixture of 1.5% isoflurane, 40% N2O and 20% O2. For each eye, local anesthesia with Oxybuprocaine hydrochloride was topically applied before injection. The needle was inserted at an angle through the sclera (to avoid damage to the lens) with the tip aimed towards the vitreous body such that the substance was uniformly distributed. The needle was kept in the eye for 30 seconds to prevent reflux and then slowly withdrawn. The contralateral eye was injected with an equal volume of saline (0.9% NaCl) and served as a control. To avoid infections, eyes were topically treated with one drop of the antibiotic Ofloxacin before and after injection. A preliminary experiment showed reduced retinal ganglion cell (RGC) survival after repeated daily injections of FC. Therefore, a single injection was used in this study.

MTT assay to assess Müller cell metabolic activity and viability

An initial series of experiments was performed to determine the optimal FC concentration for Müller cell inhibition. For this, murine primary Müller cells of C57BL/6 pups, aged 8-10 days, were cultured following the protocol from Da Silva *et al.*, (2008). Cells were incubated with FC at concentrations ranging from 0.16 to 0.64 mM. After incubation for 4, 6, 12 and 24 hours, cell viability and metabolic activity were assessed using the Cell Proliferation Kit I (MTT). Briefly, 5000 cells per well were seeded in a 96-well microplate. The cells were incubated overnight to allow them to attach. The cells were then further incubated with 10 µl FC solution or 0.9% NaCl (vehicle). After incubation, 10 µl of the MTT labeling reagent (3,[4, 5 dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, final concentration 0.5 mg/ml) was added, and the cells were incubated for four hours. All incubation steps were performed at 37°C and 10% CO2. To determine the cell viability, the MTT reaction was stopped by adding 100 µl of solubilization solution (10% SDS in 0.01 M HCl), and the absorbance was measured after complete solubilization of the released formazan crystals in a microplate reader at 550 nm, corrected against 650 nm.

Aconitase activity assay

Retinal aconitase activity after FC treatment was assessed according to the protocol from Gardner *et al.* (1994). Animals were treated with a final concentration of 0.32 mM FC and sacrificed four hours later. Retinae were removed and shock frozen. Whole retinal tissue was homogenized in buffer containing 50 mM Tris-Cl (pH 7.4) and 0.6 mM MnCl2 by brief ultrasonication (Sonoplus, Bandelin, Germany) for 5 sec and clarified by centrifugation at 10

000 x g for 5 min. Triplicates of the homogenates, consisting of a 200-µl reaction mixture containing 50 mM Tris-HCl (pH 7.4), 5 mM sodium citrate, 0.6 mM MnCl2, 0.2 mM NADP+, 1-2 units of isocitrate dehydrogenase, and 20 µg of extracted proteins were assayed for aconitase activity by measuring the rate of formation of Nicotinamide adenine dinucleotide phosphate hydrate (NADPH) from NADP+. The rate of formation was monitored by the linear increase in absorbance at 340 nm at 25°C. An initial lag in NADPH formation is seen in assays of low aconitase activity, which presumably reflects the delayed accumulation of cis-aconitate (Krebs and Holzach 1952). Thus, the linear rates starting after 40 minutes of the 90-min assay were used to determine the activity. The increase in absorbance was plotted against the time, and differences in treatment groups were confirmed by covariance analysis.

Determination of glutamine levels

The intraretinal glutamine concentration was detected using a glutamine assay kit (Abnova, Taipei City, Taiwan), which provides a quantitative colorimetric determination of glutamine at 565 nm with a detection range of 0.023 to 2 mM. Animals treated with FC were sacrificed by an overdose with 30% chloral hydrate at 3, 4, 6, 12 and 24 hours after treatment. Retinae were kept in PBS and homogenized by means of ultrasonication (Sonoplus) at minimum power for 5 sec. After centrifugation at 10 000 x g for 10 min at 4°C, the protein concentration of the supernatant was determined using the Bradford assay. The glutamine concentration was measured as indicated in the protocol provided with the kit. The glutamine concentration obtained was corrected for glutamate and for total retinal protein. The results are given as a percentage of control levels.

Induction of transient retinal ischemia

Transient retinal ischemia (TRI) was induced as described previously (Krempler *et al.* 2011; Schultz *et al.* 2016). Briefly, mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate in phosphate-buffered saline (PBS; 500 mg/kg body weight; Fluka, Seelze, Germany). Chloral hydrate was used to avoid side effects to the central nervous system. One eye was locally anesthetized with Oxybuprocaine hydrochloride followed by cannulation of the anterior chamber with a 30-gauge needle connected to a reservoir containing normal saline. To induce retinal ischemia, the intraocular pressure was increased above systolic pressure by raising the saline reservoir for 45 minutes. Increased pressure was verified by means of an Induction/Impact Tonometer (TonoLab; Tiolat Ltd., Helsinki, Finland). Then, the cannula was removed, and blood was allowed to naturally re-perfuse

the retinal tissue. Body temperature was maintained on a digitally controlled heating pad for the duration of the procedure and recovery. Animals were monitored on a daily base by the experimenter. Mice with signs of cataract were excluded from the study. The animals for FC treatment before or after ischemia were assigned at random by the principal investigator and mixed with non-injected animals.

Immunofluorescent staining

Animals were sacrificed with an overdose of chloral hydrate (30%) seven days after TRI. Eyes were enucleated and retinae flattened by making incisions from the periphery halfway to the optic nerve to form four symmetric lobes. Whole mounts were then fixed in 4% PFA for 20 min and permeabilized with 0.3% Triton X-100 in PBS for 45 min at RT. Blocking was achieved by incubation with a solution consisting of 3% bovine serum albumin and 10% normal donkey serum in PBS supplemented with 0.3% Triton X-100 for 2 hours at RT. After non-specific binding was blocked, whole mounts were incubated with a primary antibody raised against the RGC-specific transcription factor Brn3a (goat anti-Brn3a, 1:300; Santa Cruz) in 5% normal donkey serum overnight at 4°C. After washing with PBS, retinae were incubated with the corresponding secondary antibodies (Molecular Probes, Leiden, The Netherlands) in 10% normal donkey serum for one hour at RT. Specificity of the staining was tested by incubation without the primary antibody.

Retinal ganglion cell quantification

RGC survival was evaluated on retinal whole mounts seven days post ischemia. Brn3a-labeled RGCs were acquired with a laser scanning microscope (LSM 710, Zeiss, Jena, Germany) and counted in tile scans close to the optic nerve in each of the four whole-mount lobes (0.54 mm2 each, 4 fields in total). Cell density was automatically determined after image processing performed with ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij; version 1.46), following a protocol developed by Salinas-Navarro *et al.*, (2009) with some modifications. Briefly, z-stacking was performed to cover the entire thickness of the ganglion cell layer. Maximum-intensity-projected images were used for quantification. After subtracting the background, images were converted to 8-bit images, and brightness and contrast were adjusted to better visualize cells without increasing the background. Images were thresholded, and closely packed nuclei were separated with the Watershed function. The “Analyze Particles function” was then used with a minimum size of 30 µm to count cells. Parameters for cell-counting were calibrated by comparison with a subset of manually counted images.

The number of surviving RGCs was expressed as the number of cells per square millimeter, and final cell counts are given as the percentage of the corresponding contralateral and unlesioned eyes. The image acquisition and analysis was performed blinded. The whole mount preparations of animals treated with FC, non-injected ischemic controls and naïve mice were mixed randomly and coded by the principal investigator.

Quantitative polymerase chain reaction (qRT-PCR) analysis

Animals were killed with an overdose of chloral hydrate (30%) 12, 24, and 48 hours after TRI lesion. Eyes were enucleated, and retinae were shock frozen in liquid nitrogen. Isolation of mRNA was carried out using the RNeasy micro kit, and equal amounts of total retinal mRNA were reverse transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit. Quantitative RT-PCR was used to measure expression levels of the following markers: 1) the gliosis markers glial fibrillary acidic protein (*Gfap*), vimentin (*Vim*), glutamate aspartate transporter (*Glast*) and glutamine synthetase (*Glul*); 2) the inflammatory markers tumor necrosis factor alpha (*Tnf-α*), interleukin 1 beta (*Il-1β*) and cluster of differentiation 68 (*Cd68*); 3) the antioxidant enzymes superoxide dismutase 1 and 2 (*Sod1* and *Sod2*), catalase (*Cat*), glutathione peroxidase 1 (*Gpx1*) and heme oxygenase-1 (*Hmox1*), and 4) the neurotrophic factors brain-derived neurotrophic factor (*Bdnf*), glial cell line-derived neurotrophic factor (*Gdnf*), nerve growth factor (*Ngf*) and ciliary neurotrophic factor (*Cntf*). The PCR was carried out with Brilliant II SYBR Green QPCR MasterMix using the primer sequences depicted in table 1. After denaturing at 95°C for 10 minutes, 40 amplification cycles were carried out as follows: denaturation at 95°C for 60 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec. The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine–guanine phosphoribosyltransferase (*Hprt*) were used as reference genes. To determine the relative expression ratio (fold change) of the target genes, the analysis introduced by Pfaffl (2001) was applied. Analysis was repeated twice.

Statistical Analysis

All results are expressed as the mean ± standard error of the mean (SEM). Each group consisted of at least four animals. Outliers were determined using the Dixon's Q test. For cell culture experiments, the efficacy of different dosages of FC via MTT-Assay was analyzed using one way ANOVA followed by a Holm-Sidak post hoc test. Statistical significance for the aconitase activity was determined using the one-way ANCOVA test. Differences in glutamine level were analyzed using One-way ANOVA followed by Holm-Sidak post hoc

analysis. For RGC survival analysis statistical significance was assessed by using One-way ANOVA followed by Holm-Sidak post hoc analysis for comparing control mice and treatment groups. For relative gene expression ratios, One-way ANOVA followed by Holm-Sidak post hoc analysis was used for the time-dependent expression levels and Two-way ANOVA followed by Holm-Sidak post hoc analysis was applied for comparison of lesion effects 24 and 48 hours after ischemia. SigmaPlot (13.0v) was used and the level of significance and was accepted at $p<0.05$.

Results

Fluorocitrate selectively inhibited Müller cell metabolism

We first determined the effect of FC on Müller cell metabolism in the mouse retina. FC decreased the metabolic activity of cultured primary Müller cells in a dose dependent manner as determined by the MTT assay. Inhibition was already achieved four hours after adding FC to the culture medium. Metabolic activity was reduced by 20% and 23% after the addition of 0.32 and 0.64 mM FC, respectively (absorbance for 0.32 mM FC = 0.091 ± 0.004 and for 0.64 mM FC = 0.088 ± 0.003 vs. 0.114 ± 0.003 in saline-treated cells, *P<0.05*, figure 2A). A lower concentration of FC (0.16 mM) inhibited cell metabolism only after 12 hours of incubation. Inhibition by 0.64 mM FC was long-lasting, lasting up to 24 hours. To achieve a quick and short Müller cell inactivation, an FC concentration of 0.32 mM was chosen for all *in vivo* experiments. This concentration has been used in previous studies with the rat retina (Virgili *et al.* 1991).

Since FC directly inhibits the activity of the enzyme aconitase, which forms part of the Krebs cycle, we determined the activity of this enzyme after intraocular delivery of 0.32 mM FC. Aconitase activity in treated eyes was significantly lower than in untreated control eyes (figure 2B). Additionally, glutamine levels were significantly lower between three and six hours in FC-treated eyes than in untreated control eyes (39 ± 13% vs. 100 ± 23% in untreated eyes, *P<0.05*, figure 2C).

Fluorocitrate increased retinal ganglion cell survival after acute retinal ischemia/reperfusion

Next, we evaluated the effect of transient Müller cell inhibition before introducing an ischemic lesion and during the early and late reperfusion phases on RGC survival after lesioning (figure 1 and figure 3A/B).

Acute ischemia led to a high loss of RGCs seven days after lesion onset (36 ± 2% vs. 97 ± 3% in control retinae, *P<0.001*). FC delivery four hours before lesion onset had no significant effect on RGC survival (28 ± 1% vs. 37 ± 1% in untreated ischemic retinae, n.s.). FC delivery at the onset or after six hours of reperfusion also had no effect on RGC survival (reperfusion onset: 35 ± 5% vs. 37 ± 1%, n.s.; six hours reperfusion: 38 ± 3% vs. 37 ± 1%, n.s.). However, FC delivery 12 hours after lesion led to a significant increase in RGC survival seven days after lesion (66 ± 6% vs. 37 ± 1%, *P<0.001*).

Expression levels of markers for cellular stress response mechanisms were altered after transient Müller cell inhibition

To determine the mechanisms involved in neuroprotection after inhibition of Müller cell metabolism, we measured the mRNA expression levels of markers for gliosis, inflammation, antioxidant enzymes and neurotrophic factors. Detailed results are summarized in table 2.

Expression levels of gliosis markers were altered after ischemia and FC delivery

Both *Gfap* and *Vim* mRNA expression levels were significantly higher than the naïve control levels. The *Gfap* levels were highest at 48 hours after lesion onset (*P<0.001*, figure 4A). *Vim* levels were increased after lesion onset, starting at 12 hours (*P<0.001*, figure 4A). *Glast* levels first decreased at 12 hours after lesion onset but were upregulated again at 48 hours (*P<0.05* and *P<0.001*, respectively, figure 4A) compared to naïve control levels, whereas glutamine synthetase (*Glul*) was consistently downregulated starting at 12 hours (*P<0.001*, figure 4A).

FC delivery 12 hours after lesion onset had a strong effect on the expression of these markers. The *Gfap* levels 24 and 48 hours after lesion onset were lower than the *Gfap* levels in the untreated ischemic eyes (*P<0.001*, figure 4B). *Vim* expression was further increased 48 hours after lesion onset and FC injection (*P<0.001*, figure 4C). FC injection reduced the ischemia-mediated increase in *Glast* expression 24 and 48 hours after lesion onset (*P<0.001*, figure 4D). The reduction in *Glul* was less pronounced 24 hours after lesion onset (*P<0.05*) but reached the same level as that without FC treatment at 48 hours (figure 4E).

Ischemia and FC led to increased expression of inflammation-associated genes

Tnf-α expression rapidly increased and was highest after 12 hours of reperfusion (*P<0.001*, figure 5A); expression then slightly decreased up to 48 hours after lesion onset (*P<0.05*, figure 5A). A similar expression pattern was observed for *Il-1β*, with an initial strong increase at 12 hours after lesion onset (*P<0.05*, figure 5A) and a slightly reduction at 48

hours of reperfusion (*P<0.05*, figure 5A). *Cd68* expression also increased after lesion onset, starting at 12 hours (*P<0.001*, figure 5A) and was highest at 48 hours of reperfusion (*P<0.05*, figure 5A).

Tnf-α expression was further increased after FC delivery and remained elevated between 24 and 48 hours of reperfusion compared with the levels in untreated mice (*P<0.05*, figure 5B). The expression of *Il-1β* did not change after ischemia induction and FC injection (figure 5C); however, *Cd68* levels were increased at 24 and 48 hours after lesion onset in FC-injected mice compared with the levels in untreated mice (*P<0.05*, figure 5D).

Expression of antioxidant enzymes increased after FC treatment

Sod1 expression was highest at 24 hours of reperfusion and remained high 48 hours after lesion onset (*P<0.001*, figure 6A). *Sod2* expression was highest at 12 hours of reperfusion (*P<0.001*, figure 6A) but returned to the control level at 48 hours (figure 6A). The expression of *Cat* was decreased 12 hours after lesion onset (*P<0.05*) and then showed a continuous increase over control levels up to 48 hours after lesion onset (*P<0.001*, figure 6A). *Gpx1* expression peaked at 24 hours of reperfusion (*P<0.001*, figure 6A) and remained elevated until 48 hours of reperfusion. The elevation of *Hmox1* expression was highest at 12 hours of reperfusion (*P<0.001*) and remained above the control levels until 48 hours of reperfusion (*P<0.001*, figure 6A).

FC delivery starting 12 hours after reperfusion onset had a strong effect on the expression of these markers. The expression of both *Sod1* and *Sod2* was significantly increased by FC 24 and 48 hours after lesion onset compared with the levels in ischemic untreated eyes (*P<0.001*, figure 6B/C). FC blocked the increase in catalase expression 24 hours after lesion onset (*P<0.05*, figure 6D) but had no effect at 48 hours. The expression levels of *Gpx1* after FC injection were unchanged at 24 hours but significantly increased at 48 hours of reperfusion (*P<0.001*, figure 6E). After FC injection, the *Hmox1* levels were increased at 48 hours of reperfusion compared with the levels in untreated eyes (*P<0.05*, figure 6F); however, the expression levels in FC treated eyes at 48 hours of reperfusion were lower than the corresponding levels at 24 hours and followed a similar pattern as that found for untreated eyes (*P<0.05*, figure 6F).

Expression of neurotrophic factors was changed after ischemia and FC delivery

The *Bdnf* levels increased after lesion onset, with peak expression at 12 hours after ischemia induction (*P<0.05*, figure 7A), and then decreased below the basal level at 48

hours of reperfusion (*P<0.001*, figure 7A). *Gdnf* expression was also increased and was highest at 12 hours after ischemia (*P<0.001*, figure 7A), returning to the basal level at 48 hours of reperfusion. *Ngf* levels showed the same pattern of expression and were highest at 12 hours post lesion (*P<0.001*, figure 7A), reaching basal levels at 48 hours after ischemia induction. In contrast, *Cntf* mRNA levels did not change immediately after lesion onset but were significantly elevated at 48 hours post lesioning (*P<0.001*, figure 7A).

FC injection 12 hours post lesioning led to an additional increase in *Bdnf* expression at 24 hours of reperfusion (*P<0.05*, figure 7B) and a less pronounced decrease in expression at 48 hours of reperfusion (*P<0.001*, figure 7B). The same pattern of expression was observed for *Gdnf* (24 hours: *P<0.001*, 48 hours: *P<0.05*, figure 7C). Additionally, *Ngf* expression increased at 24 hours after lesion onset and FC treatment (P<0.001, figure 7D). For *Cntf*, FC injection led to a less pronounced increase at 48 hours of reperfusion compared to the increase observed in the untreated ischemic eyes (*P<0.001*, figure 7E).

Discussion

In this study, we evaluated the effect of transient inhibition of Müller cell metabolism on retinal ganglion cell survival after acute ischemia/reperfusion *in vivo*. We hypothesized that impairing Müller cell activation after an ischemic lesion induction would block the detrimental effects of the reactive gliotic response, thereby improving neuronal survival after lesioning.

Since there were no data available about the effect of FC in the mouse retina, we first evaluated its effectiveness in isolated mouse Müller cells *in vitro*. FC is a competitive inhibitor of aconitase and reversibly blocks the Krebs cycle (Clarke *et al.* 1970; Peters and Shorthouse 1972). FC reduces Müller cell metabolism in the retina of several organisms, including rats, chickens and frogs (Virgili *et al.* 1991; Zeevalk and Nicklas 1997; Jablonski and Iannaccone 2000), without adversely affecting the metabolism of neurons (Paulsen *et al.* 1987; Hassel *et al.* 1992; Hayakawa *et al.* 2010). In agreement with the aforementioned studies, FC specifically reduced Müller cell metabolism *in vitro*. *In vivo*, we found a significant reduction in aconitase activity at the FC concentration reported for the rat retina (Virgili *et al.* 1991). Similarly in agreement with previous studies, the inhibition of aconitase with FC maximally reduced retinal glutamine levels between four and six hours after

delivery (Swanson and Graham 1994; Zeevalk and Nicklas 1997). The levels were restored 24 hours after treatment.

Next, we determined the effect of Müller cell inhibition on ischemia-induced retinal ganglion cell death by delivering FC at various points in time before and after lesion onset. Delivery of FC four hours prior to induction of retinal ischemia, as well as immediately or six hours after lesion onset, did not affect neuronal survival. However, when delivered 12 hours after lesion onset, FC significantly improved neuronal survival seven days after lesioning. Our results are in agreement with a study by Andreeva *et al.* (2014) indicating the presence of different phases during ischemia/reperfusion injury in the retina. These phases might depend on the differential activation of glial cells, as suggested here. Our findings further indicate that the detrimental effects of Müller cell activation are present as early as 12-18 hours after ischemia induction.

To evaluate the possible mechanisms involved in neuroprotection induced by Müller cell inhibition after ischemia, we measured the expression levels of markers known to be involved in the retinal damage response. In accordance with previous studies, we found increased expression of the intermediate filaments *Gfap* and vimentin after lesion onset (Kim *et al.* 1998; Wurm *et al.* 2011). Intermediate filaments stabilize the hypertrophied processes of Müller cells and have been proposed to be involved in the signal transduction pathways underlying gliosis. In addition, the expression levels of *Glast*, which is involved in the glial and neuronal uptake of glutamate, were reduced during the early reperfusion phase but had increased by a later time point, whereas glutamine synthetase, a Müller cell-specific enzyme, showed a consistent decrease in expression. This is also in line with previous studies (Otori *et al.* 1994; Nishiyama *et al.* 2000; Kruchkova *et al.* 2001; Moreno *et al.* 2005). Interestingly, reduced activity of glutamine synthetase is already known to result from depressed GLAST expression, which contributes to neuronal damage following ischemia induction (Ishikawa *et al.* 2011).

Müller cells coordinate inflammation in the retina and produce pro-inflammatory cytokines, such as Il-1*β* and TNF-α which contribute to the ischemia/reperfusion injury (Wang *et al.* 2011; Li *et al.* 2012). Moreover, Müller cells interact with microglia in a bi-directional manner, which helps to shape the overall response to injury in the retina (Wang and Wong 2014). In our study, the expression levels of the pro-inflammatory cytokines *Il-1β* and *Tnf-α* as well as the microglial/macrophage activation marker *Cd68*, were significantly higher after lesion onset, which is also in agreement with previous reports (Hangai *et al.* 1995; Song *et al.* 2017; Berger *et al.* 2008; Scholz *et al.* 2015; Bae *et al.* 2016).

Ischemia/reperfusion injury is also associated with the excess generation of reactive oxygen species (ROS) (Bonne *et al.* 1998). Here, we evaluated the expression levels of antioxidant enzymes catalyzing the conversion of ROS, including *Gpx1*, *Sod1*, *Sod2*, *Cat*, and *Hmox*, all known to be involved in the response to ischemia. In agreement with other studies and our own work, the expression levels of antioxidant enzymes, with the exception of catalase, were increased in the retina early after ischemia (Arai-Gaun *et al.* 2004; Andreeva *et al.* 2014; Schultz *et al.* 2016; Agardh *et al.* 2006).The increased levels of neurotrophic factors in the central nervous system and the neuroretina after an ischemic insult have been suggested to be part of an endogenous protective response (Bringmann and Wiedemann 2011). In agreement with previous studies, we found a transient increase in *Bdnf*, *Gdnf* and *Ngf* levels following ischemia-reperfusion (Lönngren *et al.* 2006; Miyazaki *et al.* 2001; Yang and Duan 2013; Sanchez *et al.* 2003; Gustavsson *et al.* 2008).

The increase in ganglion cell survival after FC delivery found here was accompanied by changes in the expression levels of all the aforementioned markers. In particular, *Gfap* expression was significantly reduced both at 24 and 48 hours after lesion onset. Vimentin expression, by contrast, was significantly increased at 48 hours after ischemia induction. The expression of intermediate filaments in activated Müller cells normally follows the same pattern. The selective increase in vimentin might be independent of Müller cells, since reactive microglia are known to also overexpress this intermediate filament (Graeber *et al.* 1988; Jiang *et al.* 2012). Furthermore, FC reduced *Glast* expression 24 hours after lesion onset and reversed the increase observed 48 hours after ischemia induction. Importantly, in mice, GLAST takes up approximately 50% of the glutamate (Sarthy *et al.* 2005). Whether FC also affects the expression of other glutamate uptake mechanisms in the retina is not known. FC treatment also partially reversed the impaired expression of glutamine synthetase at 24 but not 48 hours after lesion onset. Changes in the glutamine synthetase expression in Müller cells under pathological conditions have been associated with changes in IL-1*β* levels (Shen and Xu 2009). Since FC did not significantly change the expression levels of *Il-1β* it is probably not involved in the FC-mediated effect on glutamine synthetase. Alternatively, neurotrophic factors such as BDNF can increase the expression of glutamine synthetase (Dai *et al.* 2012). In our study, we found increased expression of several neurotrophic factors, including BDNF (discussed below), after FC treatment, which might partially explain the glutamine synthetase increase observed here.

Since our findings point to a reduced gliotic response after transient Müller cell inhibition, we also evaluated the expression of inflammatory and microglial activation markers. The

levels of *Tnf-α* and *Cd68* but not of *Il-1β* were dramatically increased. In a similar study in rats, *Il-1β* expression was reported to peak between 3 and 12 hours after lesion onset (Hangai *et al.* 1995). This could partially explain the lack of effect on *Il-1β* after FC delivery, which showed a maximum effect 18 hours after injury. On the other hand, these data might also indicate that the regulation of *Il-1β* levels is independent of the Müller cell activation status after injury. With regard to *Tnf-α* FC delivery prolonged the lesion-induced increase in *Tnf-α* expression. TNF-α is an inflammatory cytokine that is known to increase in concentration after an ischemic injury and to induce neuronal damage (Bae *et al.* 2016). However, TNF-α has also been found to be neuroprotective against glutamate-induced excitotoxicity (Marchetti *et al.* 2004) and to protect RGCs after retinal ischemia induction and early after an optic nerve lesion. This effect is probably mediated by Müller cells (Fontaine *et al.* 2002; Mac Nair *et al.* 2014). Our findings are in line with a study by Li *et al.*, (2012) who found a similar response pattern in cultured Müller cells, with increased cell viability and reduced levels of NF-κB, IL-1*β* and Cox-2 but not TNF-α after hypoxic injury and post-lesion delivery of lutein, which was shown to have an anti-inflammatory effect.

Müller cell inhibition further increased the expression of phagocytic *Cd68* following injury. Although this finding might at first indicate an increased inflammatory response and microglial/macrophage activity after FC delivery, CD68 is expressed by microglia/macrophages in both the M1 and M2 activation states and is also present in TAMs (tumor-associated macrophages), which release many anti-inflammatory, pro-angiogenic and growth factors (Komohara *et al.* 2008; Roszer 2015). Furthermore, increased microglial/macrophage phagocytic activity has been found to be beneficial and neuroprotective after acute inflammatory processes, by removing dead neurons and clearing cell debris (Fu *et al.* 2014).

Since ischemic pathology is characterized by increased levels of oxidative stress, we evaluated the antioxidant response in the ischemic retina after FC delivery. With the exception of catalase, the expression levels of all antioxidant enzymes tested were significantly increased. Elevated GPX1 has been associated with reduced cell death after experimental stroke (Ishibashi *et al.* 2002). Increased *Hmox1* expression in Müller cells has been found to play a protective role in retinal ischemia-reperfusion injury in rats (Sun *et al.* 2007; Peng *et al.* 2008; Arai-Gaun *et al.* 2004; Ulyanova *et al.* 2001). Additionally, increased *Sod1* and *Sod2* expression has been associated with better neuronal survival after acute retinal ischemia (Liu *et al.* 2012; Schultz *et al.* 2016). These results indicate that reduced

Müller cell reactivity after ischemia induction leads to an improved antioxidant response, partially explaining the increased neuronal survival found in this study.

As already mentioned, neuronal degeneration after ischemia involves the withdrawal of neurotrophic factors. Müller cells produce and release bFGF (Wen *et al.* 1995), BDNF (Seki *et al.* 2005) and GDNF (Harada *et al.* 2002), a process modulated by microglia through the release of NGF, BDNF and CNTF (Harada *et al.* 2002). Furthermore, in the presence of activated microglia, Müller cells express higher mRNA and protein levels of trophic factors, such as GDNF and LIF (Wang *et al.* 2011), which is consistent with the pattern we found in our study. With the exception of *Cntf*, treatment with FC significantly increased the expression of growth factors 24 hours after lesion onset. Since CNTF production after retinal lesion onset is localized to Müller cells as part of the endogenous neuroprotective system in the retina (Honjo *et al.* 2000), reduced *Cntf* expression after FC delivery probably reflects the reduced metabolic and activation status of Müller cells. Importantly, extracellular glutamate can increase neurotrophic factor expression in Müller cells (Taylor *et al.* 2003). The aforementioned halt of the increase in GLAST after FC treatment could lead to increased extracellular glutamate levels. In support of this, FC, in addition to the well described decrease in glutamine levels, has been shown to significantly increase extracellular glutamate levels in the brain, and this is followed by a significant increase in NGF mRNA expression (Gwag *et al.* 1997). This FC-mediated effect is blocked by NMDA glutamate receptor antagonists and suggests that NGF mRNA expression is regulated by changes in glutamate levels and could partly explain the increase we found after ischemia and FC treatment. Whether a similar mechanism is involved in the changes observed in BDNF and GDNF needs to be further investigated. Importantly, elevated NGF expression has also been localized to microglia (Yang and Duan 2013; Sanchez *et al.* 2003). In the same studies, the increase in NGF was blocked by minocycline, which specifically inhibits microglial activity (Yang and Duan 2013).

Taking these findings together, our study shows that transient metabolic inhibition of Müller cells increases neuronal survival in the post-ischemic retina and further strengthens the idea that these cells play a central role in ischemia-associated neurodegeneration. Increased neuronal survival was associated with a reduced gliotic response and increased antioxidant defense mechanisms and neurotrophic support, thereby promoting a neuronal protective environment 12 hours after lesion onset. Our results support the notion that the detrimental effects of reactive gliosis can be inhibited to improve neuronal survival. There

are only a few reports on the reversibility of reactive gliosis in retinal degenerative conditions (Ganesh and Chintala 2011; Livne-Bar *et al.* 2016; Tura *et al.* 2009).

Whether the modulation of Müller cell metabolism will also be neuroprotective in chronic degenerative diseases such as glaucoma remains to be investigated. This study supports the concept that glial cells constitute a feasible therapeutic target to overcome neuronal damage following an acute ischemic lesion. Furthermore, we propose that a combined therapy targeting neuronal and glial elements would significantly improve neuronal survival after injury.

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Table 1. Primer sequences used in the study, together with the expected product length

			Primer sequence		Product size
Gliosis					
Gfap	fw	5'	AGAAAGGTTGAATCGCTGGA	3'	176 bp
	re	5'	GCCACTGCCTCGTATTGAGT	3'	
Vim	fw	5'	TGAAGGAAGAGATGGCTCGT	3'	194 bp
	re	5'	GGTGTCAACCAGAGGAAGTGA	3'	
Glast	fw	5'	GCCCTCCGACCGTATAAAAT	3'	128 bp
	re	5'	GCCATTCCTGTGACGAGACT	3'	
Glul	fw	5'	ATCGGGTGTGCGAAGACTTT	3'	181 bp
	re	5'	CGAATGTGGTACTGGTGCCT	3'	
Inflammation					
TNF-α	fw	5'	GTCTACTGAACTTCGGGGTGAT	3'	102 bp
	re	5'	ATGATCTGAGTGTGAGGGTCTG	3'	
Il-1β	fw	5'	GAAGAGCCCATCCTCTGTGA	3'	96 bp
	re	5'	TTCATCTCGGAGCCTGTAGTG	3'	
Cd68	fw	5'	TTCTGCTGTGGAAATGCAAG	3'	116 bp
	re	5'	GAGAAACATGGCCCGAAGT	3'	
Antioxidant enzymes					
Sod1	fw	5'	GTCCGTCGGCTTCTCGTCT	3'	163 bp
	re	5'	CACAACTGGTTCACCGCTTG	3'	
Sod2	fw	5'	ATTAACGCGCAGATCATGCA	3'	161 bp
	re	5'	TGTCCCCCACCATTGAACTT	3'	
Cat	fw	5'	GCAGATACCTGTGAACTGTC	3'	229 bp
	re	5'	GTAGAATGTCCGCACCTGAG	3'	
Gpx1	fw	5'	GGGACTACACCGAGATGAACGA	3'	197 bp
	re	5'	ACCATTCACTTCGCACTTCTCA	3'	
Hmox-1	fw	5'	GGTGATGGCTTCCTTGTACC	3'	155 bp
	re	5'	AGTGAGGCCCATACCAGAAG	3'	
Neurotrophic factors					
Bdnf	fw	5'	TGGCTGACACTTTTGACCAC	3'	131 bp

	re	5'	CAAAGGCACTTGACTGCTGA	3'	
Gdnf	fw	5'	TGGGCTATGAAACCAAGGAG	3'	142 bp
	re	5'	CAACATGCCTGGCCTACTTT	3'	
Ngf	fw	5'	AGCATTCCCTTGACACAG	3'	99 bp
	re	5'	GGTCTACAGTGATGTTGC	3'	
Cntf	fw	5'	ATGACTGAGGCAGAGCGACT	3'	157 bp
	re	5'	AGGCAGAAACTTGGAGCGTA	3'	
Housekeeping genes					
Hprt	fw	5'	TGACACTGGTAAAACAATGCA	3'	94 bp
	re	5'	GGTCCTTTTCACCAGCAAGCT	3'	
Gapdh	fw	5'	AGGTCGGTGTGAACGGATTTG	3'	123 bp
	re	5'	TGTAGACCATGTAGTTGAGGTCA	3'	

fw, forward primer; re, reverse primer; bp, base pairs.

Table 2. Expression levels of selected markers at various time points during reperfusion after transient retinal ischemia with and without FC treatment (mean ± SEM)

Gliosis marker

	Gfap	*Vim*	*Glast*	*Glul*
Control	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.04
12 h reperfusion	2.84 ± 0.29 *	4.31 ± 0.18 **	0.71 ± 0.03 *	0.44 ± 0.03 **
24 h reperfusion	5.76 ± 0.23 * +	5.58 ± 0.17 *	1.21 ± 0.04 ++	0.38 ± 0.03 *
48 h reperfusion	14.55 ± 2.74 * + §	4.09 ± 0.58 *	2.54 ± 0.29 ** ++ §§	0.42 ± 0.08 *
24 h reperfusion + FC	3.71 ± 0.18 §	5.65 ± 0.08	0.56 ± 0.04 §§	0.62 ± 0.01 §
48 h reperfusion + FC	7.71 ± 0.34 & %%	8.26 ± 0.77 && %	1.22 ± 0.06 && %%	0.34 ± 0.03 %

Inflammatory markers

	Tnf-α	*Il-1β*	*Cd68*
Control	1.01 ± 0.11	1.00 ± 0.04	1.00 ± 0.06
12 h reperfusion	18.68 ± 1.33 **	35.08 ± 2.83 *	5.04 ± 0.32 **
24 h reperfusion	23.96 ± 7.87 **	12.24 ± 2.24 * ++	6.69 ± 0.23 ** +
48 h reperfusion	10.80 ± 1.87 ** + §	8.58 ± 3.94 ** +	8.09 ± 1.05 ** +
24 h reperfusion + FC	24.2 ± 0.71	9.84 ± 3.63	10.6 ± 1.06 §
48 h reperfusion + FC	26.1 ± 2.21 &	5.64 ± 0.56	12.9 ± 0.78 &

Antioxidant enzymes

	Sod1	*Sod2*	*Cat*	*Gpx1*	*Hmox1*
Control	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.04
12-h reperfusion	1.11 ± 0.03 *	1.29 ± 0.04 **	0.78 ± 0.07 *	1.20 ± 0.02 *	19.33 ± 3.53 **
24 h reperfusion	1.33 ± 0.02 ** ++	1.27 ± 0.03 **	1.18 ± 0.05 * ++	1.77 ± 0.05 ** ++	11.97 ± 2.84 **
48 h reperfusion	1.19 ± 0.02 ** §	0.97 ± 0.01 ++ §§	1.45 ± 0.04 ** ++ §	2.00 ± 0.15 ** ++	4.86 ± 1.09 ** ++ §
24 h reperfusion + FC	1.52 ± 0.03 §§	1.41 ± 0.01 §§	1.04 ± 0.03 §	1.58 ± 0.04	17.97 ± 0.7
48 h reperfusion + FC	1.67 ± 0.04 && %	1.20 ± 0.02 && %%	1.44 ± 0.06 %%	3.04 ± 0.11 && %%	9.72 ± 0.90 § %

Neurotrophic factors

	Bdnf	*Gdnf*	*Ngf*	*Cntf*
Control	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.04
12 h reperfusion	1.39 ± 0.04 *	5.78 ± 0.53 **	2.04 ± 0.18 **	0.99 ± 0.08

24 h reperfusion	1.25 ± 0.05 *	1.81 ± 0.37*++	1.29 ± 0.04 +	1.20 ± 0.05
48 h reperfusion	0.60 ± 0.05 ** ++ §	0.91 ± 0.07 ++ §	1.12 ± 0.13 ++	3.15 ± 0.25 ** ++ §§
24 h reperfusion + FC	1.78 ± 0.18 §	7.36 ± 0.75 §§	2.91 ± 0.37 §§	1.43 ± 0.13
48 h reperfusion + FC	1.03 ± 0.04 && %%	1.44 ± 0.22 & %%	1.40 ± 0.17 %	1.65 ± 0.08 &&

** *P<0.001,* * *P<0.05* vs. naïve control
++ *P<0.001,* + *P<0.05* vs. 12 hours of reperfusion
§§ *P<0.001,* § *P<0.05* vs. 24 hours of reperfusion
&& *P<0.001,* & *P<0.05* vs. 48 hours of reperfusion
%% *P<0.001,* % *P<0.05* vs. 24 hours reperfusion with FC

Figure legends

Figure 1. Experimental design for intravitreal FC administration and metabolic inhibition of Müller cells from female C57BL/6 mice *in vivo*. Mice were sacrificed 12, 24 and 48 hours after treatment for qRT-PCR analysis or after 7 days to quantify surviving RGCs after lesion.

Figure 2. Efficacy of different dosages of FC *in vitro* and *in vivo*. **A**) MTT assay with cultured primary Müller cells. Absorbance was assayed 4, 6, 12 and 24 hours after FC treatment. Bars represent the mean ± SEM**.** One-way ANOVA. **P*<0.05 vs. Müller cellsl cells treated with saline; n = 6. **B**) Aconitase activity 4 hours after intravitreal injection of FC or saline. Aconitase activity was determined over a period of 50 min. Values were corrected against blank measurements. Measurement points represent the mean ± SEM. One-way ANCOVA, *P*<0.05, n = 4. **C**) Time-dependent effects of FC on glutamine levels in the retina. Glutamine levels are expressed as percentage of those in naïve control eyes. One-way ANOVA, *P*<0.05 vs. control. Bars represent the mean ± SEM**,** n = 5.

Figure 3. Effect of FC treatment on RGC survival 7 days after acute retinal ischemia/reperfusion. **A**) Retinal whole-mount preparations from unlesioned, lesioned and FC-treated animals. Images were taken near the optic nerve by means of laser scanning microscopy (Zeiss LSM 710). Scale bars represent 50 µm. **B**) Analysis of surviving RGCs after acute retinal ischemia. Numbers of RGCs are expressed as percentages of contralateral eyes. Cell counts were determined 7 days after ischemia in retinae treated with FC at different points in time before and after lesion onset (FC -4 h: treatment 4 hours before lesion onset, FC 0 h: treatment at reperfusion onset, FC +6 h/FC +12 h: treatment at late reperfusion. One-way ANOVA ****P*<0.001 vs. unlesioned control, +++ P<0.001 vs. FC + 12 h; Bars represent the mean ± SEM, n = 5.

Figure 4. Effect of acute retinal ischemia/reperfusion and FC treatment on mRNA levels of gliosis markers. **A**) Expression levels of corresponding mRNAs were determined 12, 24 and 48 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt*. Logarithmic scaling; bars represent the mean ± SEM, n = 4. One-way ANOVA, *** *P*<0.001; * *P*<0.05. **B-E**) Effects of FC on *Gfap*, *Vim*, *Glast* and *Glul* expression levels are depicted as the ratio of treated vs. naïve corresponding control eyes. mRNA levels were determined 24 hours and 48 hours after ischemia and FC treatment. Bars represent the mean ± SEM, n = 4, Two-way ANOVA, *** *P*<0.001; * *P*<0.05.

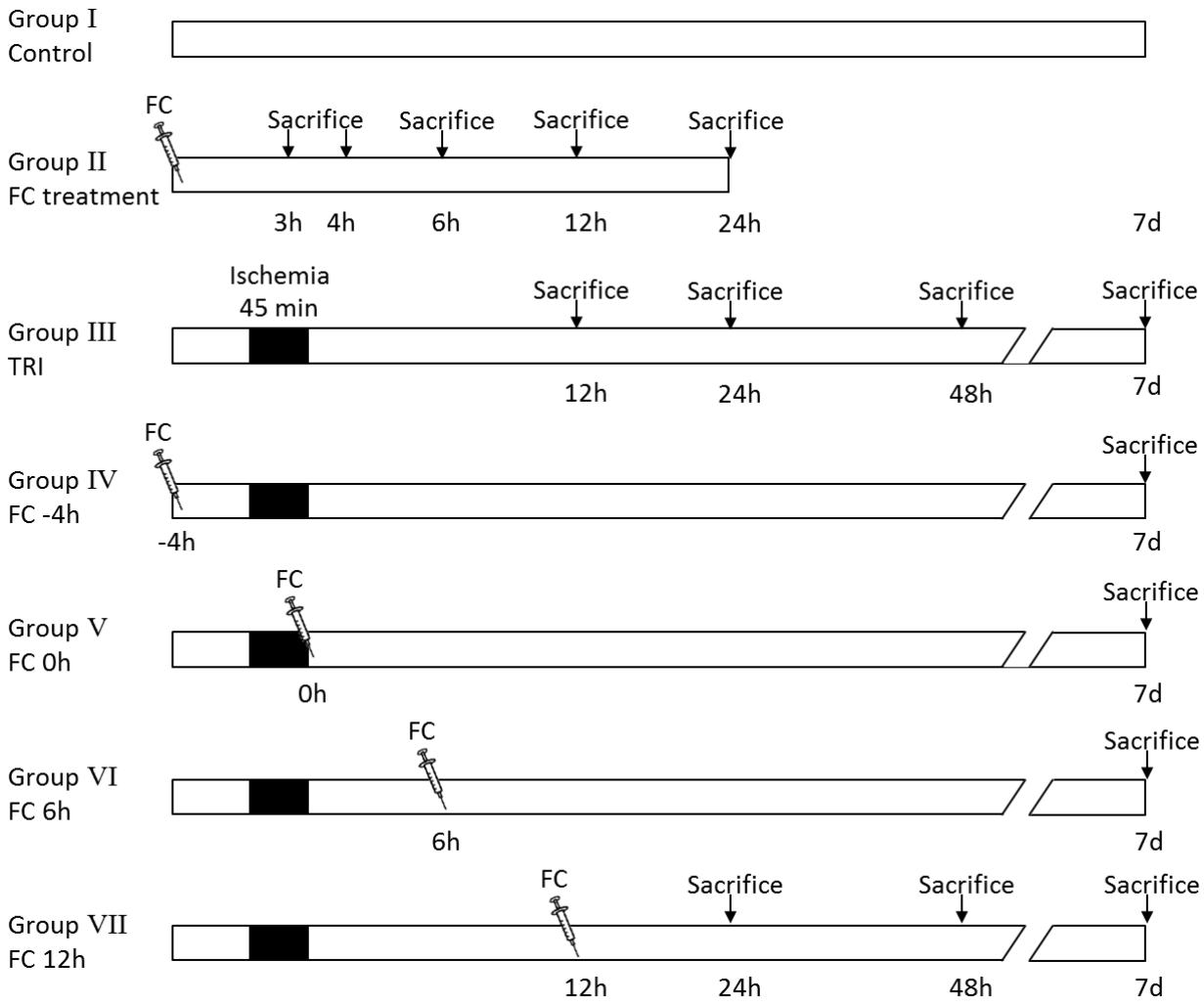
Figure 5. Effect of acute retinal ischemia/reperfusion and FC treatment on mRNA levels of inflammatory markers. **A**) Expression levels of corresponding mRNA were determined 12,

24 and 48 hours after ischemia by quantitative polymerase chain reaction and normalized to *Gapdh* and *Hprt*. Logarithmic scaling, bars represent the mean ± SEM, n = 4. One-way ANOVA, *** *P*<0.001; * *P*<0.05. **B-D**) Effects of FC on *Tnf-α*, *Il-1β*, and *Cd68* expression levels are depicted as the ratio of treated vs. naïve corresponding control eyes. mRNA levels were determined 24 hours and 48 hours after ischemia and FC treatment. Bars represent the mean ± SEM, n = 4. Two-way ANOVA, *** *P*<0.001; * *P*<0.05.

Figure 6. Effect of acute retinal ischemia/reperfusion and FC treatment on mRNA levels of antioxidant enzymes. A) Expression levels of corresponding mRNAs were determined 12, 24 and 48 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt*. Logarithmic scaling for *Hmox1*; bars represent the mean ± SEM, n = 4. One-way ANOVA, *** *P*<0.001; * *P*<0.05. B-F) Effects on *Sod1*, *Sod2*, *Cat*, *Gpx1* and *Hmox1*, expression are depicted as the ratio of treated vs. naïve corresponding control eyes. mRNA levels were determined 24 hours and 48 hours after ischemia and FC treatment. Bars represent the mean ± SEM, n = 4. Two-way ANOVA, *** *P*<0.001; * *P*<0.05.

Figure 7. Effect of acute retinal ischemia/reperfusion and FC treatment on mRNA levels of neurotrophic factors. **A**) Expression levels of corresponding mRNAs were determined 12, 24 and 48 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt*. Bars represent the mean ± SEM, n = 4. One-way ANOVA, *** *P*<0.001; * *P*<0.05. **B-F**) Effects of FC on *Bdnf*, *Gdnf*, *Ngf* and *Cntf* expression are depicted as the ratio of treated vs. naïve corresponding control eyes. mRNA levels were determined 24 hours and 48 hours after ischemia and FC treatment. Bars represent the mean ± SEM, n = 4. Two-way ANOVA, *** *P*<0.001; * *P*<0.05.

Figure 1


Group I
Control
FC
Sacrifice
Sacrifice
Sacrifice
Sacrifice
Group II
FC treatment
3h
4h
6h
12h
24h
7d
Ischemia
45 min
Sacrifice
Sacrifice
Sacrifice
Sacrifice
Group III
TRI
12h
24h
48h
7d
FC
Sacrifice
Group IV
FC -4h
-4h
7d
FC
Sacrifice
Group V
FC 0h
0h
7d
FC
Sacrifice
Group VI
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Group VII
FC 12h
12h
24h
48h
7d

Figure 2

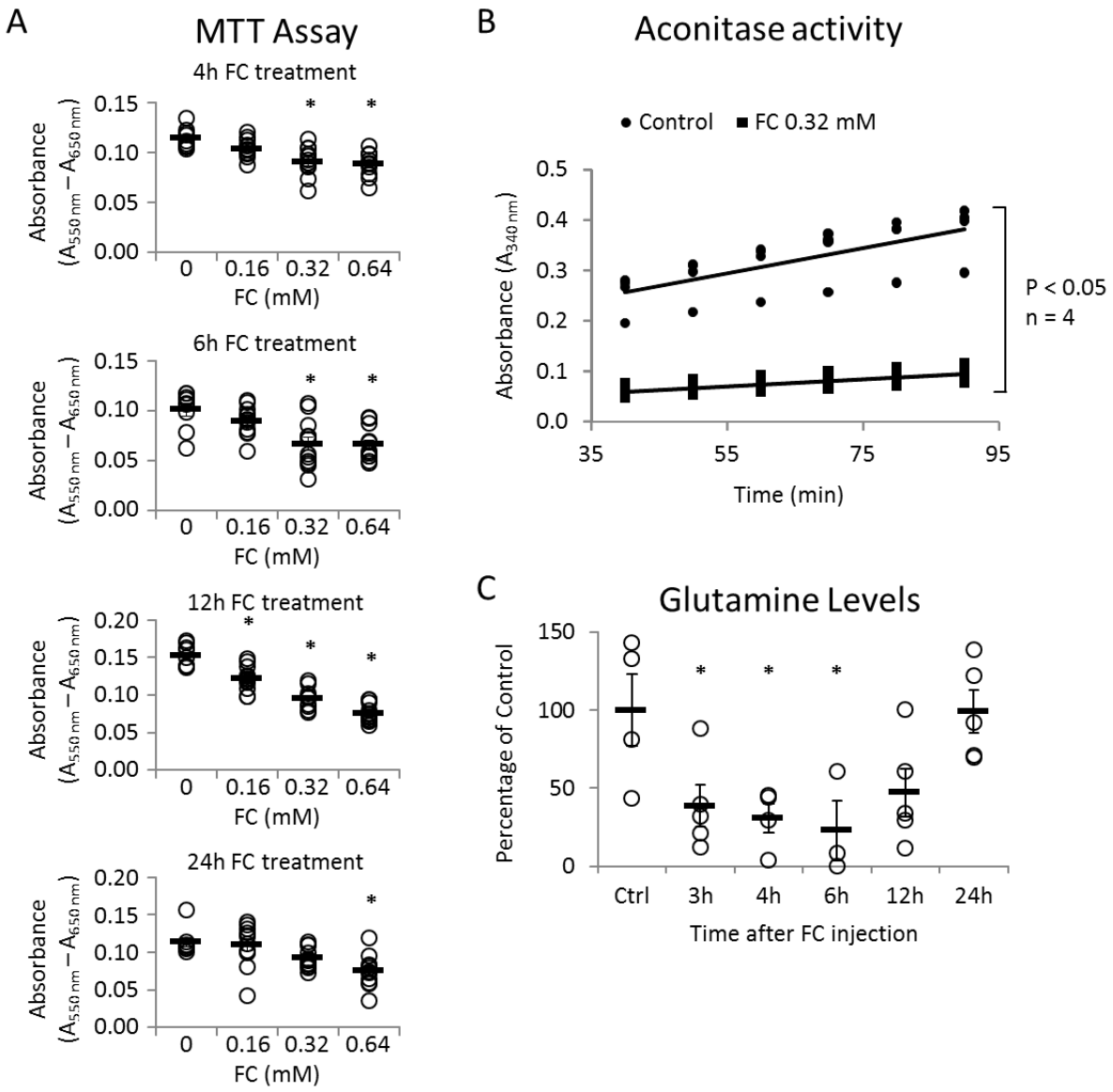

A
MTT Assay
4h FC treatment
6h FC treatment
12h FC treatment
24h FC treatment
Absorbance ($A_{550\,nm} - A_{650\,nm}$)
FC (mM)
0
0.16
0.32
0.64
B
Aconitase activity
Control
FC 0.32 mM
Absorbance ($A_{340\,nm}$)
Time (min)
P < 0.05
n = 4
C
Glutamine Levels
Percentage of Control
Ctrl
3h
4h
6h
12h
24h
Time after FC injection

Figure 3

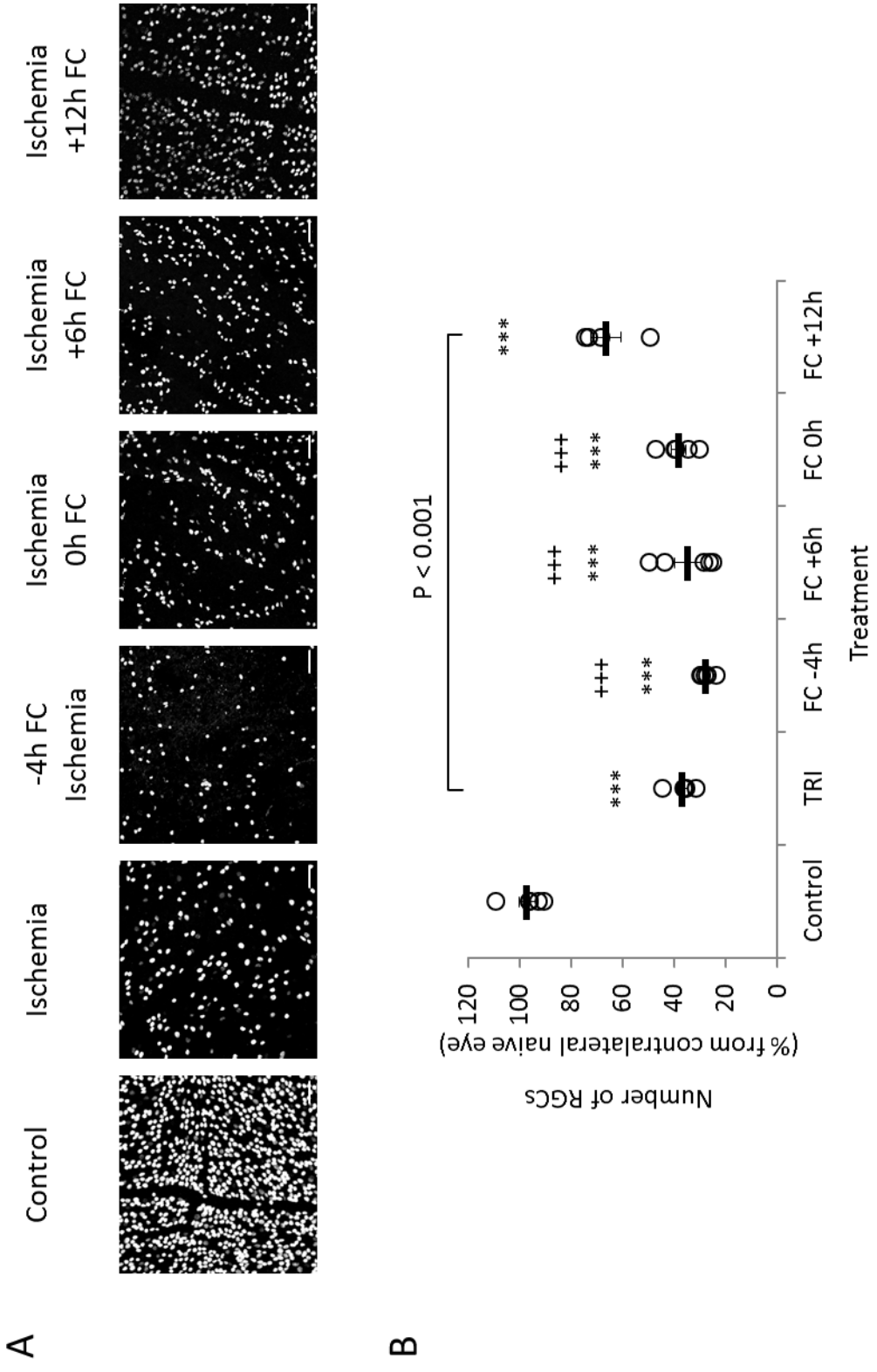
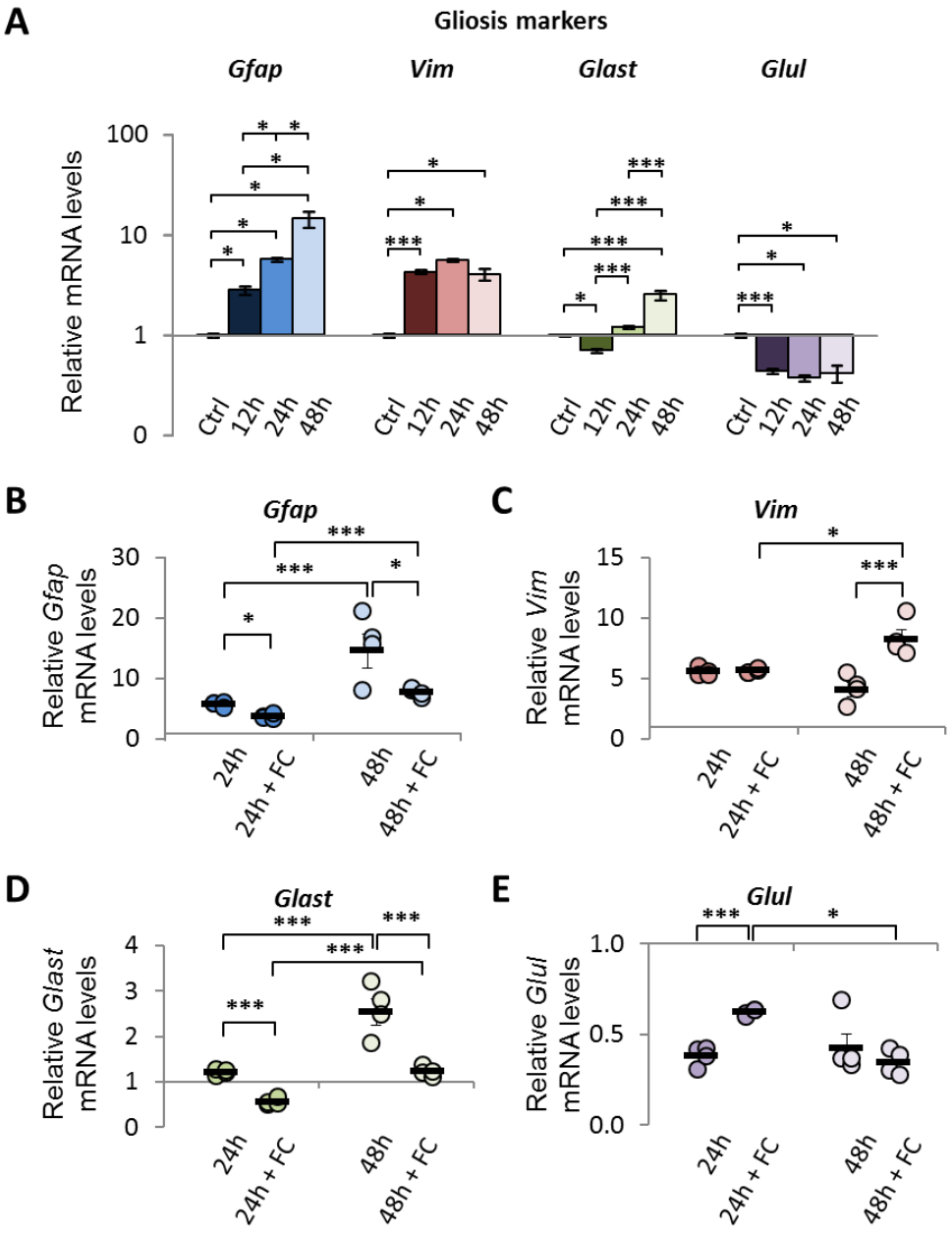

A
Control
Ischemia
-4h FC Ischemia
Ischemia 0h FC
Ischemia +6h FC
Ischemia +12h FC
B
Number of RGCs (% from contralateral naive eye)
P < 0.001
***
+++
Control
TRI
FC -4h
FC +6h
FC 0h
FC +12h
Treatment

Figure 4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Gliosis markers
Gfap
Vim
Glast
Glul
Relative mRNA levels
100
10
1
0
Ctrl
12h
24h
48h
B
Gfap
Relative Gfap mRNA levels
30
20
10
0
C
Vim
Relative Vim mRNA levels
15
10
5
0
D
Glast
Relative Glast mRNA levels
4
3
2
1
0
E
Glul
Relative Glul mRNA levels
1.0
0.5
0.0
24h
24h + FC
48h
48h + FC

Figure 5

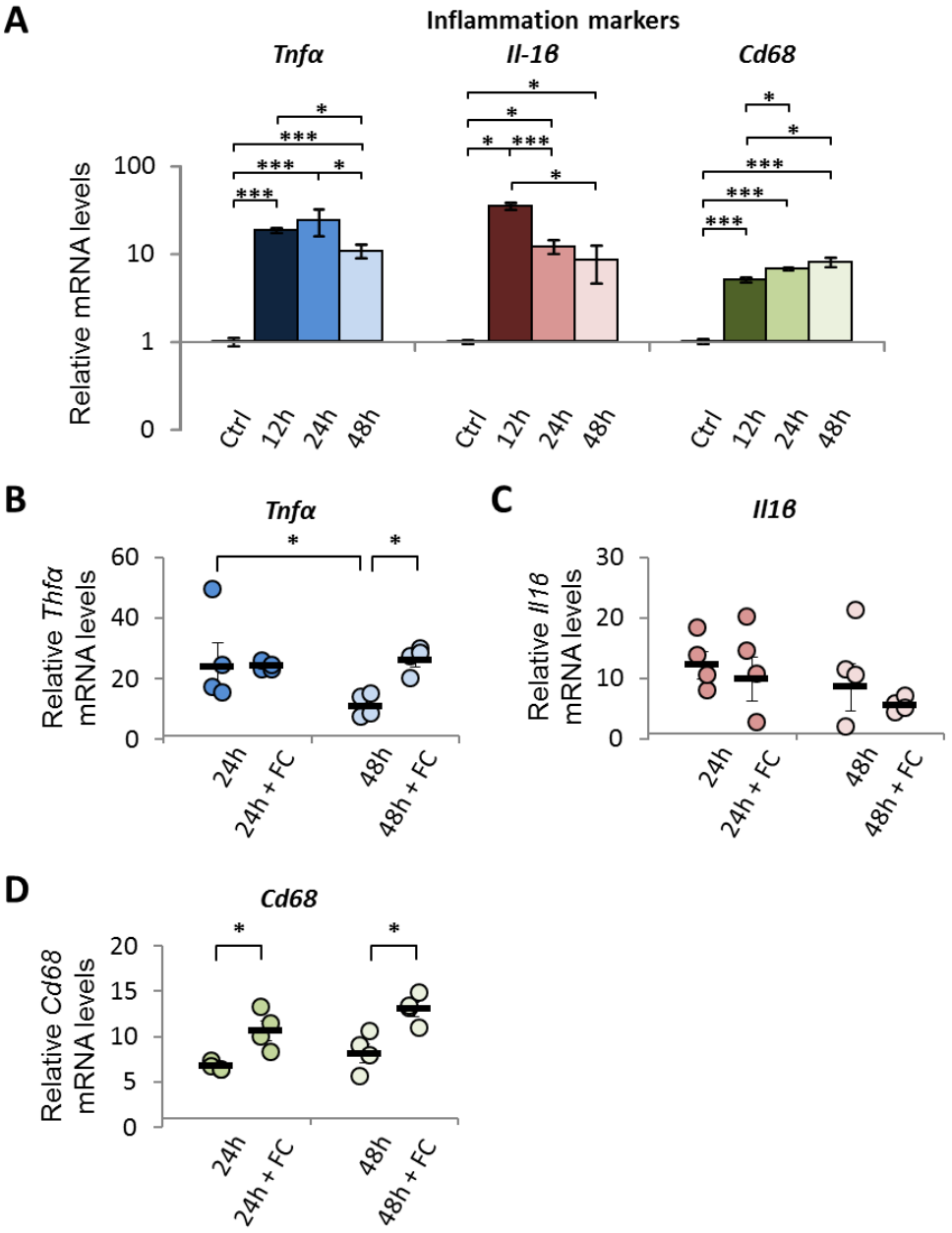

A
Inflammation markers
Tnfα
Il-1β
Cd68
Relative mRNA levels
100
10
1
0
Ctrl
12h
24h
48h
B
Tnfα
Relative Thfα mRNA levels
60
40
20
0
24h
24h + FC
48h
48h + FC
C
Il1β
Relative Il1β mRNA levels
30
20
10
0
D
Cd68
Relative Cd68 mRNA levels
20
15
10
5
0

Figure 6

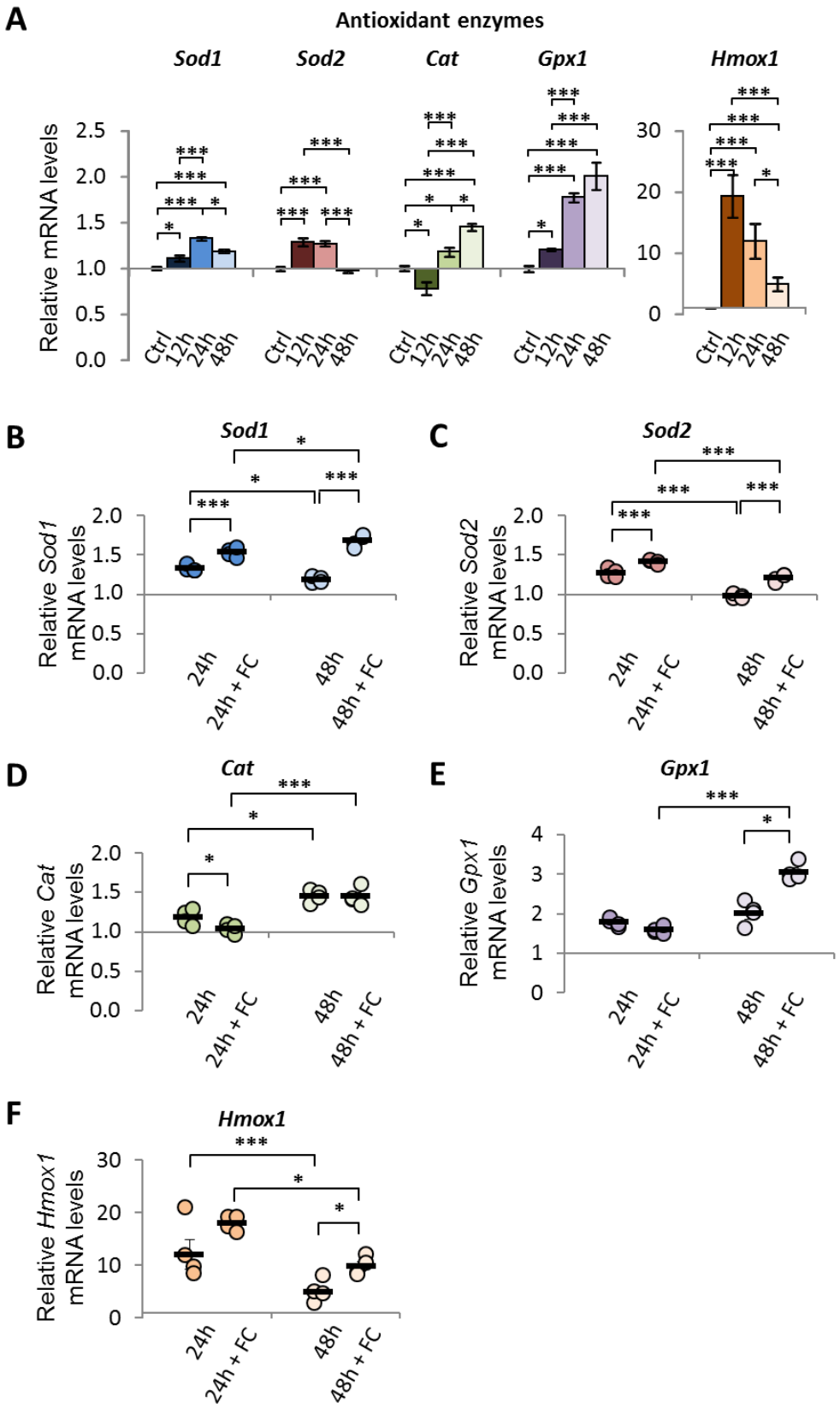
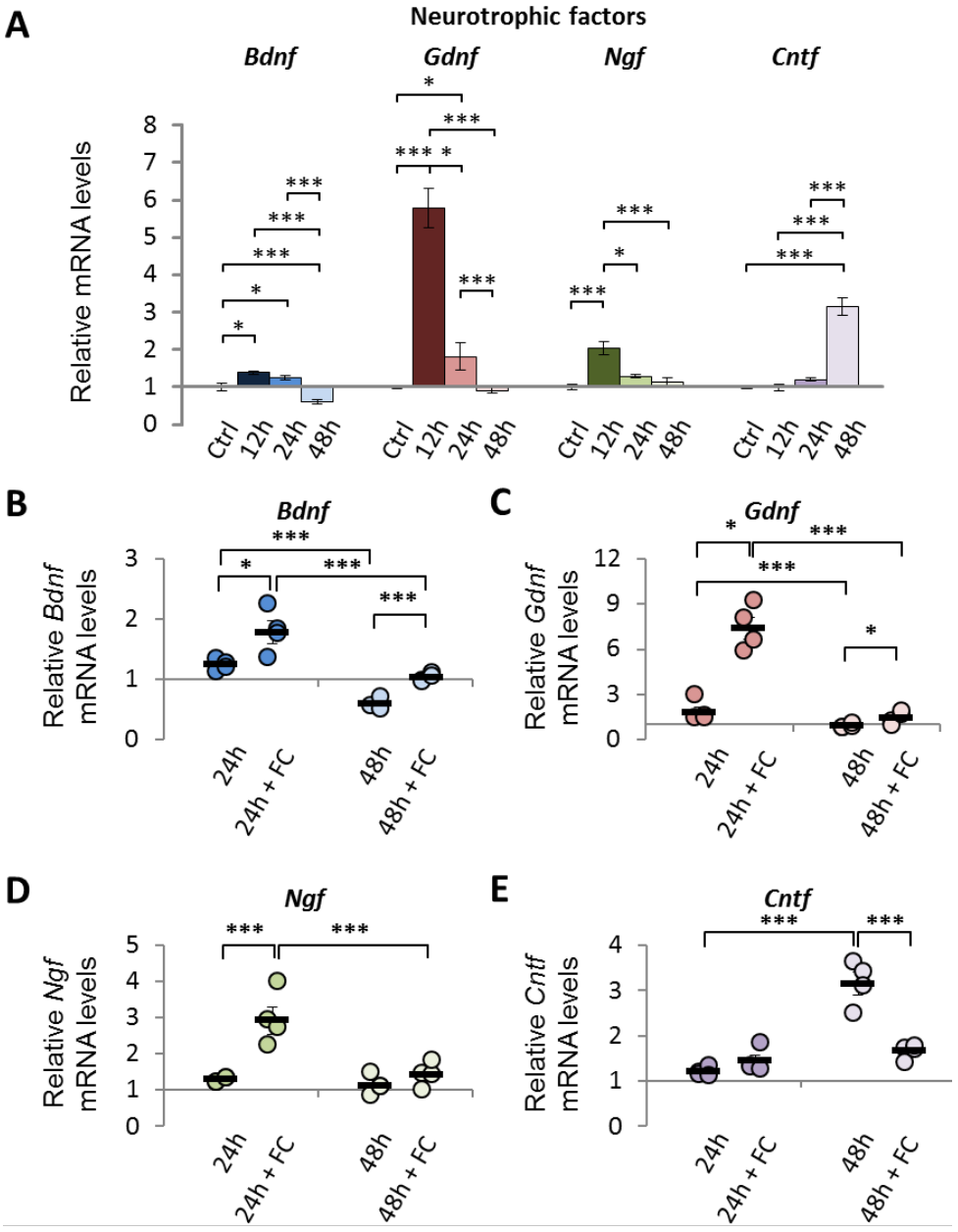

A
Antioxidant enzymes
Sod1
Sod2
Cat
Gpx1
Hmox1
Relative mRNA levels
Ctrl
12h
24h
48h
B
Sod1
Relative Sod1 mRNA levels
C
Sod2
Relative Sod2 mRNA levels
D
Cat
Relative Cat mRNA levels
E
Gpx1
Relative Gpx1 mRNA levels
F
Hmox1
Relative Hmox1 mRNA levels
24h
24h + FC
48h
48h + FC

Figure 7


A
Neurotrophic factors
Bdnf
Gdnf
Ngf
Cntf
Relative mRNA levels
Ctrl
12h
24h
48h
B
Bdnf
Relative Bdnf mRNA levels
C
Gdnf
Relative Gdnf mRNA levels
D
Ngf
Relative Ngf mRNA levels
E
Cntf
Relative Cntf mRNA levels
24h
24h + FC
48h
48h + FC

4.3 Manuscript III

Journal: Scientific Reports

Title: Frataxin overexpression in Müller cells protects retinal ganglion cells in a mouse model of acute glaucoma *in vivo*

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Abstract

Müller cells are critical for retinal function and neuronal survival but can become detrimental in response to retinal ischemia and increased oxidative stress. Elevated oxidative stress increases expression of the mitochondrial enzyme frataxin in the retina, and its overexpression is neuroprotective after ischemia. Whether frataxin expression in Müller cells might improve their function and protect neurons after ischemia is unknown. The aim of this study was to evaluate the effect of frataxin overexpression in Müller cells on neuronal survival after retinal ischemia/reperfusion in the mouse *in vivo*.

Retinal ischemia was induced in mice overexpressing frataxin in Müller cells by transient elevation of intraocular pressure. Retinal ganglion cells survival was determined 14 days after lesion. Expression of frataxin, antioxidant enzymes, growth factors and inflammation markers was determined with qRT-PCR, Western blotting and immunohistochemistry 24 hours after lesion.

Following lesion, there was a 65% increase in the number of surviving RGCs in frataxin overexpressing mice. Improved survival was associated with increased expression of the antioxidant enzymes Gpx1 and Sod1 as well as the growth factors Cntf and Lif. Additionally, microglial activation was decreased in these mice.

Therefore, support of Müller cell function constitutes a feasible approach to reduce neuronal degeneration after ischemia.

Introduction

Glaucoma is the leading cause of irreversible blindness, and due to the complexity of pathological mechanisms involved, there are no clinically effective treatments available so far. One key element of the pathologic alteration in glaucoma and retinal ischemia/reperfusion injury is the generation of excessive reactive oxygen species (ROS) during reperfusion. Retinal glia (i.e., Müller cells and astrocytes) play a fundamental role in maintaining redox homeostasis and are equipped with tools to resolve redox imbalance[1,2]. Müller cells are the predominant glia in the vertebrate retina, expand the entire retina forming a structural scaffolding and have cell contacts to all neuronal cell types in the different retinal layers. Therefore they can form a functional unit with a more critical role than for astrocytes, which are restricted to the nerve fiber layer[1]. After injury, Müller cells become reactive and participate in glial scar formation. Interestingly, metabolic support of

neurons by Müller cells has been shown to be compatible, even with proliferative reactive gliosis induced in mice[3]. However, morphological and functional alterations in the MG have been found under ischemic conditions or elevated levels of oxidative stress[4], which probably compromise their supportive role.

Mitochondria are the main source of free radical production in the cell, under both, normal and ischemic conditions[5,6]. Following ischemic injury, endogenous mechanisms of cellular defense are activated to minimize damage. These include superoxide dismutases (SODs), glutathione, vitamins A, C, and E, catalase and other peroxidases. Moreover, recent evidence supports a role for the mitochondrial protein frataxin (FXN) in various diseases, which are caused by increased oxidative stress. FXN deficiency is closely associated with increased oxidative stress damage in Friedreich ataxia (FRDA), an autosomal recessive disease characterized by progressive neurodegeneration in the spinal cord and hypertrophic cardiomyopathy[7–9]. FXN acts as an important regulator of mitochondrial energy metabolism[10,11] and is involved in several important functions including cellular iron homeostasis and redox balance during oxidative stress7. The mature FXN protein is highly expressed in mitochondria of metabolically active tissues such as liver, skeletal and cardiac muscle, and brain[12]. In the adult mouse retina, immunoreactivity to FXN has been shown in the external and internal plexiform layers, the ganglion cell layer and the internal nuclear layer. Highest levels of FXN were observed in internal segments of the photoreceptor cells[13].

Improving essential functions of glia was shown to augment glial support to retinal neurons1. In particular, support of the glial management of retinal redox homeostasis has been proposed as a therapeutic alternative to overcome neuronal degeneration due to excessive oxidative stress[1]. Because Müller cells span the entire retina and quickly respond to injury, they are ideal targets for therapeutic strategies, including gene therapy[14]. Delivery of exogenous antioxidants or the manipulation of antioxidant pathways within the retina favors the interaction between retinal glia and neurons, and improves RGC survival and function[1,15]. In particular, FXN overexpression was found to stimulate the production of ATP and to induce the activation of antioxidant mechanisms8. In a previous study, we found an increased expression of endogenous FXN in the mouse retina 24 hours after an ischemic lesion, indicating that FXN is involved in neuroprotective mechanisms in response to ischemic retinal injury[16]. Furthermore, in the same study, we showed that ubiquitous overexpression of human FXN under the transcriptional control of a human cytomegalovirus (CMV) minimal promoter resulted in neuronal protection. Neuroprotection was associated with changes in expression levels of some stress response molecules,

including *Hmox1* and *Hif-2α* which suggested the participation of glial cells, particularly Müller cells, in FXN-mediated neuroprotection. To further evaluate the cellular mechanisms involved in the FXN-mediated neuroprotection in the ischemic retina and the putative role of Müller cells, here we used a conditional transgenic mouse model overexpressing the human FXN gene specifically in these cells.

Results

FXN expression was increased in the retina of naïve MGCre-FXN mice and specifically localized to Müller cells

To evaluate the effect of FXN overexpression in Müller cells on neuronal survival after ischemia, we generated transgenic mice specifically expressing the human FXN cDNA and EGFP transcript in Müller cells (referred to as MGCre-FXN mice, see materials and methods).

Quantification of mRNA for both, murine and human FXN, showed specific expression of the human FXN transcript in the retina of FXN overexpressing mice (Fig. 1A) and increased levels for endogenous mouse FXN compared with MGCre-B6 mice (1.00 ± 0.05 vs. 1.50 ± 0.11-fold increase, $P < 0.001$). The Western blot analysis showed higher levels of expression for both, precursor (30 kDa) and mature FXN protein (18 kDa) in MGCre-FXN mice, compared to MGCre-B6 mice (45% and 65% increase respectively, $P<0.05$, Fig. 1B). However, human FXN protein was indistinguishable from the endogenous mouse variant (Fig. 1B').

To prove the cellular localization of human FXN, retinal slices were co-stained with anti-GFP and the Müller cell-specific marker glutamine synthetase (GLUL). EGFP was specifically localized in cells spanning the whole retina from FXN overexpressing mice (Fig. 1C). The expression of EGFP was co-localized with the specific Müller cells marker GLUL in the retina from the MGCre-FXN mice (Fig. 1C-C'') and was detected in 41 ± 8% of all retinal Müller cells.

Gene expression of oxidative stress-related markers was altered in naïve MGCre-FXN mice

First, we evaluated whether FXN overexpression leads to changes in gene expression in naïve MGCre-FXN mice. In the study we focused on markers well known to be involved in the retinal response and neuronal protection after lesion. These include neurotrophic factors such as leukemia inhibitory factor (*Lif*), nerve growth factor (*Ngf*), brain-derived

neurotrophic factor (*Bdnf*), ciliary neurotrophic factor (*Cntf*) and glial cell line-derived neurotrophic factor (*Gdnf*), antioxidant enzymes including glutathione peroxidase 1 (*Gpx1*), superoxide dismutase [Cu-Zn] (*Sod1*), superoxide dismutase [Mn] (*Sod2*), catalase (*Cat*) and heme oxygenase 1 (*Hmox-1*), gliosis and inflammation markers including vimentin (*Vim*), glial fibrillary acidic protein (*Gfap*), glutamate aspartate transporter (*Glast*), glutamine synthetase (*Glul*), tumor necrosis factor alpha (*Tnf-α*) and Interleukin 1 beta (*Il-1β*). We measured the relative mRNA expression by means of qRT-PCR and compared the values against those obtained in naïve non-transgenic MGCre-B6 mice.

The expression levels of the growth factor *Bdnf* were significantly increased, whereas mRNA levels of *Cntf* and *Lif* were decreased in naïve MGCre-FXN mice compared with control MGCre-B6 mice (*Bdnf*: 1.22 ± 0.02-fold increase, $P < 0.001$; *Cntf*: 0.83 ± 0.01-fold decrease, $P < 0.001$; *Lif*: 0.41 ± 0.02-fold decrease, $P < 0.05$, Fig. 2). The inflammation marker *Tnf-α* was significantly decreased in naïve transgenic retinae (0.55 ± 0.06-fold decrease, $P < 0.05$, Fig. 2) and the antioxidative enzyme *Cat* was significantly increased (1.16 ± 0.07-fold increase, $P < 0.05$, Fig. 2). Furthermore, the glia marker *Vim*, which is upregulated during gliosis, was decreased in naïve MGCre-FXN mice (0.66 ± 0.05 vs. 1.00 ± 0.03-fold decrease, $P < 0.05$, Fig. 2). The other markers tested did not differ in naïve MGCre-FXN compared with MGCre-B6 mice.

FXN overexpression in Müller cells increased RGC survival after ischemia

We next examined whether the expression of FXN in Müller cells increased survival of RGCs after acute transient retinal ischemia. In naïve retinae from both, MGCre-FXN and MGCre-B6 mice, RGC distribution was highest around the optic nerve (1/6 from retinal radius) and decreased towards the periphery (5/6 from retinal radius). No differences in the overall cell distribution were observed between strains (Fig. 3A/B, Table 2).

After lesion, the number of RGCs decreased in MGCre-B6 and MGCre-FXN mice to 35 ± 2% ($P < 0.001$; $n = 6$) and 58 ± 6% ($P < 0.001$; $n = 8$) respectively, compared with non-lesioned control eyes (Fig. 3B'). This represents an increased RGC survival in mice overexpressing FXN of 65% 14 days after ischemia compared with MGCre-B6 mice ($P < 0.05$, Fig. 3B').

Interestingly, as illustrated in Fig. 4A, the retinal thickness was increased in non-lesioned MGCre-FXN mice compared with control animals. In particular, thickness of the GCL/IPL, as well as the INL, was increased by 24% and 13%, respectively. This was accompanied by a slight, but significant increase of basal IOP in naïve MGCre-FXN mice (16 ± 0.3 mmHg vs. 14 ± 0.3 mmHg, $P < 0.05$, Fig. 4B).

Retinal ischemia significantly reduced the thickness of the GCL/IPL by approximately 20% on average in MGCre-FXN mice compared with MGCre-B6 animals. Despite an increased neuronal survival, no differences in retinal thickness were found between strains after injury (Fig. 4A/C).

Increased expression of antioxidant enzymes in MGCre-FXN mice after ischemia

Gene expression ratios of the antioxidant enzymes *Gpx1*, *Sod1* and *Sod2*, *Cat* and *Hmox1* were evaluated 24 hours after ischemia by means of qRT-PCR to determine whether increased antioxidative capacity might be involved in FXN-mediated neuroprotection.

The expression levels of all enzymes evaluated were significantly increased in MGCre-B6 mice after lesion compared to basal levels (*Gpx1*: 1.60 ± 0.05-fold increase, P<0.001; *Sod1*: 1.16 ± 0.03-fold increase, P<0.001; *Sod2*: 1.20 ± 0.02-fold increase, P<0.001; *Cat*: 1.31 ± 0.02-fold increase, P<0.001; *Hmox1*: 9.44 ± 0.72-fold increase, P<0.001).

All tested markers also increased above basal levels in MGCre-FXN mice after lesion, (*Gpx1*: 1.89 ± 0.05-fold increase, P<0.001; *Sod1*: 1.33 ± 0.06-fold increase, P<0.001; *Sod2*: 1.16 ± 0.05-fold increase, P<0.001; *Cat*: 1.33 ± 0.03-fold increase, P<0.001; *Hmox1*: 9.44 ± 1.56-fold increase, P<0.001). In addition, the expression levels of *Gpx1* and *Sod1* were significantly higher in MGCre-FXN mice compared with the MGCre-B6 animals after injury (P<0.05; Fig. 5A).

Expression of neurotrophic factors is altered in MGCre-FXN mice after lesion

To evaluate the role of growth factors in FXN-mediated neuroprotection, changes in expression of the growth factors *Gdnf*, *Ngf*, *Bdnf*, *Cntf* and *Lif* were analyzed 24 hours after ischemia by means of qRT-PCR.

The expression levels of *Ngf*, *Bdnf*, *Cntf* and *Lif* were significantly increased in MGCre-B6 mice (*Ngf*: 1.60 ± 0.11-fold increase, P < 0.001; *Bdnf*: 1.43 ± 0.03-fold increase, P<0.001; *Cntf*: 1.39 ± 0.09-fold increase, P < 0.001; *Lif*: 8.44 ± 0.65-fold increase, P<0.001).

In MGCre-FXN mice, all measured factors were also increased above basal levels 24 hours after ischemia (*Ngf*: 1.59 ± 0.16-fold increase, P<0.001; *Bdnf*: 1.36 ± 0.03-fold increase, P<0.001; *Cntf*: 1.8 ± 0.16-fold increase, P < 0.001; *Lif*: 4.71 ± 1.06-fold increase, P < 0.001).

Moreover, *Cntf* expression was higher and *Lif* expression was lower in MGCre-FXN mice compared with MGCre-B6 animals (P < 0.05; Fig. 5B).

Expression levels of inflammation and gliosis marker are not different between MGCre-FXN and MGCre-B6 mice after lesion

Expression of the cytokines *Tnf-α* and *Il-1β* and of the gliosis marker *Glast*, *Vim*, *Glul* and *Gfap* were evaluated 24 hours after ischemia by means of qRT-PCR to determine whether inflammation or an altered gliotic response is involved in FXN-mediated neuroprotection.

Pro-inflammatory cytokines were significantly increased in MGCre-B6 mice after lesion compared to basal levels (*Tnf-α*: 6,20 ± 0.42-fold increase, P<0.001; *Il-1β*: 1.73 ± 0.21-fold increase, P<0.001). The expression levels of the intermediary filaments *Gfap* and *Vim* were increased after lesion, as well as the mRNA levels of *Glast* (*Gfap*: 14.04 ± 0.97-fold increase, P<0.001; *Vim*: 5.47 ± 0.31-fold increase, P<0.001; *Glast*: 1.50 ± 0.08-fold increase, P<0.001). *Glul* levels decreased after ischemia (0.28 ± 0.01-fold decrease, P<0.001).

In MGCre-FXN mice, inflammatory markers were also increased above basal levels after lesion (*Tnf-α*: 6,82 ± 0.97-fold increase, P<0.001; *Il-1β*: 1.62 ± 0.48-fold increase, P<0.001). Expression of the intermediary filaments *Gfap* and *Vim* as well as the levels of *Glast* were increased after injury (*Gfap*: 18.03 ± 2.97-fold increase, P<0.001; *Vim*: 4.63 ± 0.53-fold increase, P<0.001; *Glast*: 1.49 ± 0.12-fold increase, P<0.001). *Glul* levels decreased after ischemia (0.30 ± 0.04-fold decrease, P<0.001).

No differences in the expression of these markers were detected between MGCre-B6 and MGCre-FXN mice after ischemia.

Microglia response was impaired in MGCre-FXN mice after lesion

Müller cells and microglia communicate via bidirectional signaling that can mediate adaptive responses within the retina following injury[17]. To determine whether FXN expression in Müller cells might influence the microglia response, we evaluated the distribution of Iba1+ microglia and expression of the activation markers CD68 and MHCII in retinal slices before and after lesion. CD68 is a lysosomal protein and can be used to stain microglia in the retina. High expression levels are associated with macrophages and reactive microglia, while low levels of expression are associated with quiescent ramified microglia[18–20]. In addition, reactive microglia express the major histocompatibility complex class II (MHCII) for antigen presentation[21,22].

No differences in the number of Iba1+ microglia were observed in naïve retinae of MGCre-B6 and MGCre-FXN mice (16 ± 2 and 14 ± 1 cells/1000µm, respectively, P < 0.001, Fig. 6 and Fig.7A). Few CD68 (4.5 ± 1% and 8.3 ± 1%, respectively) positive and almost no MHCII positive microglia were found (2.8 ± 1% and 4.1 ± 1%, respectively, Fig. 6 and Fig. 7).

After ischemia, the percentage of Iba1+/Cd68+ microglia strongly increased in both, MGCre-B6 and MGCre-FXN mice; however, in MGCre-FXN mice the number of CD68+

microglia was lower compared with MGCre-B6 mice (56 ± 9% vs. 81.1 ± 1%; P<0.05; Fig. 7B). The number of MHCII+ microglia after lesion increased only in MGCre-B6 mice (7.8 ± 2% vs. 2.8 ± 1%; P<0.05; Fig. 7C).

Discussion

In the present work, we generated a mouse model overexpressing human FXN specifically in retinal Müller cells. Naïve transgenic mice showed slightly decreased expression of gliosis and inflammation markers and of neurotrophic factors. After ischemia, we found an increased RGC survival in transgenic mice overexpressing FXN. Improved cell survival correlated with an increase in gene expression of antioxidant enzymes and neurotrophic factors, whereas the activity of retinal immune cells (microglia) was reduced.

In FXN overexpressing mice the expression levels of hFXN mRNA and FXN protein levels were significantly elevated in lysates from the whole retina. These data are similar to results obtained in a previous study using a transgenic mouse model with ubiquitous FXN in all retinal cells[16]. FXN overexpression was specifically increased in about 41% of all Müller cells. This value is similar to data shown in a previous study using the same Cre-recombinase expressing mice model[23]. Naïve transgenic animals did no show differences in the number or distribution of RGCs and microglia as compared with MGCre-B6 animals; however, morphological analysis of the naïve retina from FXN overexpressing mice showed an overall increase in retinal thickness, mainly manifested in the GCL and IPL but also in the INL. We also found a slight but significant increase in basal IOP levels in these animals. Different IOP levels ranging from an IOP of 11.1 ± 0.5 mmHg to 19.3 ± 0.3 mmHg have been described in several different mouse strains, and have not been associated to morphological changes or development of glaucoma[24].

Retinae without Müller cells have decreased resistance to tensile stress rendering the retinal tissue to rip apart, a defect known as retinoschisis. FXN overexpression, seems to induce a similar effect by reducing the tensile strength of Müller cells, resulting in expanded retinal layers. The underlying mechanism is unknown, but could involve reduced levels of the intermediate filament vimentin and slight increased IOP levels found in these animals. Vimentin deficiency and mechanical stress have already been associated with a local separation of the inner limiting membrane leading to an increased retinal thickness[25].

There are only a few reports on transgenic mice strains overexpressing FXN and its associated effects. Mice expressing the full-length human FXN cDNA are normal with no

signs of ataxia or other obvious abnormalities[11,26]; however, these mice have an altered response during hematopoietic differentiation[26], The retina was not evaluated in that mouse model. For FXN overexpression, the existing literature presents contradictory results. On the one hand, experiments *in vivo* or *in vitro* in mice revealed that FXN overexpression was innocuous or had a positive effect on cell metabolism, stimulating the production of ATP or activating antioxidant mechanisms[11,16,26–28]. In addition, overexpression of FXN in Drosophila promoted cellular resistance to oxidative stress[8]. On the other hand, FXN overexpression can lead to detrimental phenotypes in Drosophila, including developmental defects, a decrease in the level of aconitase activity, hypersensitivity to oxidative stress[29], and reduced viability and impaired embryonic development of muscles and the peripheral nervous system[30]. Interestingly, overexpression of FXN in yeast has also been shown to critically reduce aconitase activity, leading to impaired [Fe-S] cluster assembly and respiration[31]. The mechanisms responsible for these effects are not known, but FXN aggregation and a misfolding of the protein have been shown to not be the cause for the phenotypes that have been observed[30]. Importantly, also in yeast, frataxin overproduction increased cellular antioxidant defenses[31]. In our study, FXN overexpression in Müller cells led to changes in the basal gene expression of several factors. Increased *Cat* might improve retinal homeostasis by providing a less oxidative environment, as also suggested by decreased *Lif* expression[28]. BDNF levels are known to be associated with increased expression of antioxidant proteins and reduced oxidative stress levels[32,33]. Therefore, increased *Bdnf* levels in naïve FXN overexpressing mice might also be indicative of decreased ROS levels, even before lesion onset.

Transient retinal ischemia leads to a high loss of RGCs in MGCre-B6 mice, comparable to values obtained in a previous study from our lab using C57BL/6J mice[16]. Here, we show for the first time that specific FXN overexpression in Müller cells leads to a significant increase in neuronal survival after lesion. Interestingly, we achieved the same level of neuroprotection compared with transgenic mice with ubiquitous FXN overexpression in the retina[16]. This strongly indicates that Müller cells play a critical role in neuroprotection after the ischemic lesion.

One of the hallmarks of acute retinal ischemia/reperfusion is the generation of excessive ROS leading to neuronal cell death. Müller cells are well known to protect neuronal cells from oxidative stress by providing antioxidants such as glutathione[34]. Since protective effects mediated by FXN overexpression are known to also involve several antioxidant mechanisms[26,28], we tested the effect of increased FXN expression on levels of antioxidant enzymes, including *Gpx1*, *Sod1*, *Sod2*, *Cat*, and *Hmox1*. The expression of all enzymes was

increased in the retinae of both MGCre-B6 and MGCre-FXN mice after ischemia, in agreement with other studies[2,35,36]. Importantly, *Gpx1* and *Sod1* were expressed more highly in transgenic mice than in MGCre-B6 mice. Both factors are already described to be neuroprotective[37,38]. These results are also in agreement with our previous study involving FXN overexpression in the retina, where we found increased levels of *Gpx1*, *Sod1*, *Sod2* and *Cat* transcripts after lesion[16]. This supports our previous findings showing that FXN mediates neuroprotection after ischemia by promoting the antioxidant response. To evaluate further mechanisms involved in neuroprotection by FXN expression in Müller cells, we measured the expression levels of growth factors, including *Bdnf*, *Gdnf*, *Ngf*, *Lif* and *Cntf*. Neurotrophic factors are known to have neuroprotective properties[39] and have been suggested to be part of an endogenous response mechanism after an ischemic insult[40]. In agreement with other studies, we found an increase in *Bdnf*, *Ngf*, *Lif* and *Cntf* levels following ischemia-reperfusion in both MGCre-B6 and FXN overexpressing mice[41–45]. Interestingly, in our study, only *Cntf* increased significantly over nontransgenic levels after lesion. CNTF has been described to be neuroprotective for photoreceptor cells[46–48] and RGCs in several studies[49–52]. In the retina, CNTF is expressed by various cells and particularly by Müller cells[53]. Increased *Cntf* transcripts strongly support an important role of Müller cells in FXN-mediated neuroprotection. In contrast, *Lif* transcripts did not reach the expression level of nontransgenic animals after lesion. However, it was significantly increased above transgenic basal levels. LIF is an important endogenous neuroprotective factor in the retina[54,55]. Specifically, photoreceptor injuries induce a subset of Müller cells to express Lif54. Further, it's transcript seems to be stabilized by H2O256. Together with the increased antioxidative response, these results indicate that FXN potentiates Müller cell-mediated neuroprotection after acute retinal ischemia/reperfusion by increasing the neurotrophic support.

Our results show a decreased microglia response after transient ischemia in transgenic mice overexpressing FXN in Müller cells. In models of RGC damage, the extent of microgliosis negatively correlates with the number of surviving RGCs[57]. A decreased number of microglia would be expected with increased neuronal survival; however, we did not observe differences in microglial numbers after lesion in nontransgenic and transgenic mice. Interestingly, the number of reactive microglia, as indicated by the expression of CD68 and MHCII, was decreased in FXN overexpressing mice after ischemia. CD68 and MHCII are markers of microglia activation[19–22,58]. Microglia reactivity and inflammation are common hallmarks of a broad spectrum of retinal diseases and contribute to RGC loss associated with ON damage in glaucoma[59] and retinal ischemia[60]. Blocking microglia reactivity is known

to be neuroprotective in experimental glaucoma[61–63]. Müller cells and microglia actively cross-talk to maintain constant levels of neurotrophic factors important for retinal physiology[64,65]. Microglia can directly trigger the release of several neurotrophic factors from Müller cells, including GDNF, LIF, CNTF and NGF[17,54,66,67]. Furthermore, vimentin expression serves as a marker for activated states of microglia[18,19,68]. In our study, FXN-overexpressing Müller cells seemed to regulate microglial function under physiological conditions, as suggested by reduced vimentin expression in naïve retinae. We propose that FXN-mediated modulation of the Müller cell response to lesion impacts microglial reactivity, possibly by shifting the microglial activation from a reactive M1-state to a more protective M2-state[69].

Taken together, our results show that FXN overexpression in Müller cells improves RGC survival after acute ischemia/reperfusion injury and this is associated with an increased expression of antioxidant enzymes and neurotrophic factors. Furthermore, our study highlights Müller cells as important players in retinal pathologies and indicates that support of the glial management of retinal redox homeostasis might constitute a therapeutic alternative to overcome neuronal degeneration due to increased oxidative stress.

Materials and Methods

Animal Guidelines

All experiments were performed in accordance with the European Convention for Animal Care and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were also approved by the local animal care committee (Thüringer Landesamt für Verbraucherschutz (TLV) - Abteilung 2 Gesundheitlicher und technischer Verbraucherschutz, accreditation numbers: 02-047/08 and 02-013/11). Animals were housed in standard cages in groups of 5 animals on a 14-hour light/10-hour dark cycle, with food and water available ad libitum, a temperature ranging from 22°C to 25°C and humidity ranging from 55% to 60%.

Male C57BL/6J background and transgenic mice overexpressing FXN, weighing 25 ± 2 g, aged 12 ± 1 weeks, were used in the study. Transgenic animals overexpressing FXN in Müller cells were generated by breeding homozygous mice carrying cassettes of human PVMD2-rtTA and TRE-cre (PVMD2TREcre[23,70,71], provided by Dr. Yun-Zheng Le, Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, USA) to heterozygous mice carrying a CMV-driven human FXN cDNA preceded by a loxP-flanked

stop cassette (R26CMVFxn/wt, provided by Prof. Michael Ristow, Department of Health Sciences and Technology, Swiss Federal Institute of Technology, Zürich, Switzerland), hereafter referred to as MGCre-FXN. Cre-mediated excision of the stop cassette leads to expression of the FXN gene and of green fluorescent protein (EGFP), as has been shown in our previous work with ubiquitous FXN overexpressing mice16, Background mice were generated by breeding C57BL/6J with PVMD2TREcre mice, hereafter referred to as MGCre-B6. Mice were randomized for each experimental setup into a non-lesioned group (MGCre-B6 n = 22; MGCre-FXN n = 22) or an ischemia group (MGCre-B6 n = 16; MGCre-FXN n = 18).

Transient Retinal Ischemia

Transient retinal ischemia (TRI) was performed as previously described[16,72]. Briefly, mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate in phosphate buffered saline (PBS; 500 mg/kg body weight; Fluka, Seelze, Germany). After application of topical anesthesia (4 mg/ml oxybuprocaine-hydrochloride; Bausch & Lomb GmbH, Feldkirchen, Germany), the anterior chamber of the right eye was cannulated with a 30-gauge needle connected to an elevated normal saline reservoir. Intraocular pressure (IOP) was elevated above systolic pressure (increased from 15.5 ± 2.6 mmHg to 93 ± 4.4 mmHg) for 45 minutes. IOP was measured using an induction/impact tonometer (TonoLab; Tiolat Ltd., Helsinki, Finland). This method has been validated for the mouse eye[73]. One drop of antibiotic solution (ofloxacin; Bausch & Lomb GmbH, Berlin, Germany) was applied topically to the treated eye after cannulation. After 45 minutes of ischemia, the needle was withdrawn and the IOP normalized. Treated eyes were inspected daily and animals with signs of inflammation or iatrogenic cataract were excluded from the study. Animals for RGC evaluation were sacrificed with an overdose of chloral hydrate (30%) 14 days after ischemia, as previously described16. For microglia analysis, mice were sacrificed 7 days after lesion, coinciding with highest microglial proliferation as already reported[57]. For qRT-PCR analysis, mice were sacrificed 24 hours post ischemia as described in a previous study[16].

Retinal tissue fixation and sectioning

To localize human FXN (hFXN) expression and evaluate microglia response after ischemic injury, retinal sections from background and MGCre-FXN mice were prepared. Briefly, animals were sacrificed with an overdose of chloral hydrate (30%), eyes were removed and fixed in 4% PFA for 20 min at room temperature (RT), enucleated, again fixed in 4% PFA for 20 min, washed in PBS and cryoprotected by immersion in 30% sucrose in PBS overnight at

4°C. Eyecups were then frozen in an appropriate embedding medium (Tissue Tek; Sakura, Loeterwoude, The Netherlands) and cryosectioned into 16 µm slices.

Retinal whole mounts of MGCre-B6 and MGCre-FXN mice were prepared to evaluate RGC survival following ischemia. Eyes were enucleated and retinae were removed and fixed by immersion in 4% PFA for 20 min. After washing in PBS, retinae were flattened by making incisions from the periphery halfway to the optic nerve to form four symmetric lobes.

Immunofluorescent staining

Immunofluorescent staining protocols were similar for retinal slices and whole mounts. Retinal sections were dried at 37°C for 45 min and fixed with 4% PFA for 20 min. Retinal whole mounts were permeabilized with 0.3% Triton X-100 in PBS for 45 min at RT. Blocking was achieved by incubation with 3% bovine serum albumin (BSA) and 10% normal donkey serum (NDS) in PBS supplemented with 0.3% Triton X-100 for 2 hours at RT. After non-specific binding was blocked, whole mounts were incubated with primary antibody raised against the RGC-specific transcription factor Brn3a (goat anti-Brn3a, 1:300; Santa Cruz, Heidelberg, Germany), whereas slices were incubated with primary antibodies directed against ionized calcium-binding adaptor molecule (rabbit anti-Iba1, 1:500; Wako, Neuss, Germany), cluster of differentiation 68 (rat anti-CD68, 1:200; Serotec, Düsseldorf, Germany), major histocompatibility complex class II (rat anti-MHCII, 1:10,000; affymetrix, CA, USA), GFP (goat anti-GFP, 1:100, Acris, Herford, Germany) and glutamine synthetase (mouse anti-GLUL, 1:250, Millipore, Darmstadt, Germany) in 5% NDS, overnight at 4°C. After washing with PBS, probes were incubated with corresponding secondary antibodies (Molecular Probes, Leiden, The Netherlands) in 10% NDS for one hour at RT. For nucleic acid staining, the retinal slices were immersed into a DAPI (4'-6-Diamidino-2-phenylindole) solution for 5 min. Specificity of the staining was tested by incubation without primary antibody.

Fluorescence microscopy

RGC survival was evaluated on retinal whole mounts 14 days post-ischemia. Brn3a-labeled RGCs were counted in single fields at three different retinal eccentricities in each of the four whole mount lobes (1/6, 3/6 and 5/6 from retinal radius; 0.093 mm2 each, 12 fields in total) by means of fluorescence microscopy according to Schmeer et al. (2008)[74]. The number of surviving RGCs was expressed as the number of cells per square millimeter and cell counts were given as the percentage of corresponding contralateral and non-lesioned eyes.

The number of GFP+ Müller cells specifically expressing FXN was determined on slices from MGCre-FXN mice by counting GLUL+GFP+ cell processes located in the IPL. The results are given as percentage from the whole GLUL+ cell population.

The number of Iba1+ microglia was determined on retinal sections. Cell counts were corrected for slice length and expressed as cell count per 1000 µm. The number of reactive microglia was assessed by counting Iba1+CD68+ and Iba1+MHCII+ cells.

Image acquisition and cell counting on retinal slices was performed by confocal laser scanning microscopy using the ZEN software (40x magnification, 710 Meta; Carl Zeiss Meditec, Jena, Germany).

Quantitative polymerase chain reaction analysis

Eyes were enucleated 24 hours post ischemia and retinae were shock frozen in liquid nitrogen. Isolation of mRNA was carried out using the QIAzol standard protocol (Qiagen, Hilden, Germany). Equal amounts of total retinal mRNA were reversely transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Schwerte, Germany). qRT-PCR amplification for murine (*mFxn*) and human FXN (*hFXN*), antioxidant enzymes, growth factors and inflammatory cytokines (primer sequences are depicted in Table 1) were carried out with Brilliant II SYBR Green QPCR Mastermix (Agilent Technologies, Santa Clara, CA, USA). After denaturing at 95°C for 10 min, 40 amplification cycles were carried out as follows: denaturation at 95°C for 60 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) were used as housekeeping genes. Relative expression ratios (fold changes) of the target genes were calculated using the Pfaffl method[75].

Western blotting

To evaluate the protein levels of retinal FXN in MGCre-B6 and MGCre-FXN mice, Western blotting was performed as previously described[16]. Primary antibodies raised against FXN (1:500; Santa Cruz), β-Actin (1:10,000; abcam, Cambridge, UK) and horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution) were used. Protein bands were visualized using an enhanced chemiluminescence reaction kit (Immun-Star WesternC Chemiluminescence Kit, BioRad, München, Germany), photographed with Fujifilm LAS-3000 Imager and analyzed with ImageJ software (1.46v). Measurements were done in duplicate. Each band was normalized against the corresponding β-actin band. Changes in protein expression were expressed as a percentage of control mice.

Statistical Analysis

All results are expressed as the mean ± standard error of the mean (SEM). Each group consisted of at least four animals. Proof of normal distribution was done for all data by a Shapiro-Wilk test. For RGC survival analysis, statistical significance was assessed by using one-way ANOVA followed by Holm-Sidak post hoc analysis and comparing MGCre-B6 control mice and treatment groups. For relative gene expression analysis, ratio data were log2 transformed and two-way ANOVA followed by Holm-Sidak post hoc analysis was applied for comparison of lesion effects between mice strains. For microglia cell counts and reactivity marker expression two-way ANOVA followed by Holm-Sidak post hoc analysis was applied for comparison of lesion effects between mice strains. For Western blot analysis, the results were compared by one-way ANOVA followed by Holm-Sidak post hoc analysis comparing background and transgenic mice. SigmaPlot (13.0v) was used and the level of significance was set at $P < 0.05$.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

The Contribution of each author are as follows: study design (C.S.), experimental work (R.S.), data acquisition (R.S., M.K., M.P., S.G.W.), data analysis (R.S.), manuscript discussion (O.W.W.), manuscript preparation (R.S., C.S.). All authors reviewed the manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. Expression of the human FXN transcript in the retina from MGCre-FXN mice. (A) Relative expression of human FXN (*hFXN*) mRNA was determined by quantitative polymerase chain reaction, normalized against *Gapdh* and *Hprt* mRNA levels and depicted as fold increase of expression in MGCre-B6 mice. The y-axis is depicted in logarithmic scaling. Bars represent the mean ± SEM; n = 6. (B) FXN protein levels of precursor FXN form (30 kDa) and mature FXN form (18 kDa) were determined by densitometric analysis of Western blotting in MGCre-B6 and MGCre-FXN mice. Values are expressed as a percentage of MGCre-B6 mice and normalized against β-actin. Bars represent the mean ± SEM; n = 4. (B') Representative Western blot showing FXN precursor and mature bands for FXN in MGCre-B6 and MGCre-FXN mice. Full-length blot is presented in Supplementary Figure 1. (C-C'') GFP expression in MGCre-FXN mice. Immunofluorescent staining of GFP (green), which is co-expressed with hFXN protein, and the Müller cell-specific marker glutamine synthetase (GLUL, red), as well as DAPI nuclear staining (blue) of retinal sections, were used to illustrate the hFXN location in Müller cells. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Scale bar = 50 µm.

Figure 2. Effect of FXN overexpression on mRNA levels of the neurotrophic factors *Lif*, *Cntf* and *Bdnf*, tumor necrosis factor alpha (*Tnfα*), catalase (*Cat*) and vimentin (*Vim*) in the naïve mouse retina. Bars represent the mean ± SEM, dashed lines represent 1.0 ratio, n = 5, two-way ANOVA.

Figure 3. Effect of FXN overexpression in Müller cells on RGC survival 14 days after acute retinal ischemia/reperfusion. (A) Images of retinal whole-mount preparations from lesioned and non-lesioned MGCre-B6 and MGCre-FXN animals at various retinal eccentricities (1/6, 3/6, 5/6 from retinal radius) are shown. Images were taken with a fluorescence microscope (x40). Scale bars = 50 µm. (B) Analysis of RGCs distribution per retinal eccentricity in non-lesioned MGCre-B6 and MGCre-FXN animals. Numbers of RGCs are expressed as cells/mm², n = 4. (B') Analysis of surviving RGCs after acute retinal ischemia. Numbers of RGCs are expressed as percentages from contralateral eyes. Cell counts were determined 14 days after the insult. Bars represent the mean ± SEM; n = 4 – 8. *** $P < 0.001$ vs. MGCre-B6 Ctrl; +++ $P < 0.001$ vs. MGCre-FXN Ctrl.

Figure 4. Effects of FXN overexpression on IOP and retinal morphology before and after acute ischemia/reperfusion. (A) Analysis of retinal layer thickness in naïve mice and after acute retinal ischemia. Bars represent the mean ± SEM; n = 5. *** $P < 0.001$ vs. MGCre-B6

Ctrl; +++ P < 0.001 vs. MGCre-FXN Ctrl. (B) IOP measurement before and during increased IOP. Bars represent the mean ± SEM; MGCre-B6 n = 16, MGCre-FXN n = 50; Mann-Whitney U Rank Sum Test. (C) Representative sections through both control and ischemic retinae from MGCre-B6 and MGCre-FXN mice are illustrated. After ischemia, retinal layers, particularly the IPL, are severely shrunk. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Scale bar = 50 µm.

Figure 5. Effect of acute retinal ischemia/reperfusion and FXN overexpression on mRNA levels of (A) antioxidant enzymes *Gpx1* and *Sod1* and (B) neurotrophic factors *Cntf* and *Lif*. Expression levels of corresponding mRNAs were determined 24 hours after ischemia by quantitative polymerase chain reaction and normalized against *Gapdh* and *Hprt*. Bars represent the mean ± SEM, dashed lines represent 1.0 ratio, n = 5, two-way ANOVA.

Figure 6. Distribution and localization of reactive microglia in the retina from MGCre-B6 and MGCre-FXN mice. Immunofluorescent staining for Iba1 (red), CD68 (cyan) or MHCII (cyan) as well as DAPI nuclear staining (blue) in retinal sections. Example sections of MGCre-B6 (A-C) and MGCre-FXN mice retinae (D-F) before and after lesion are shown. Iba1+ microglia (arrowheads) and Iba1+ co-localized with CD68 (notched arrows) or MHCII (circles) were observed. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Scale bar = 50 µm. (A'-C') and (D'-F') show enlarged squares containing example microglia with different merged channels for MGCre-B6 and MGCre-FXN mice. From top to bottom: DAPI; DAPI + CD68 or MHCII; DAPI + Iba1; Iba1 + CD68 or MHCII. Scale bar = 25 µm.

Figure 7. Effect of FXN on the expression of microglial reactivity markers. (A) Iba1+ cell counts per retinal section (1000 µm in length) in naïve and lesioned MGCre-B6 and MGCre-FXN mice. Bars represent the mean ± SEM, n = 5, two-way ANOVA. (B) Percentage of CD68 positive Iba1 cells in naïve and lesioned MGCre-B6 and MGCre-FXN mice. Bars represent the mean ± SEM, n = 5, two-way ANOVA. (C) Percentage of MHCII positive Iba1 cells in naïve and lesioned MGCre-B6 and MGCre-FXN mice. Bars represent the mean ± SEM, n = 5, two-way ANOVA.

Tables

Table 1: RGC quantification in murine retinal whole mount preparations as a function of the retinal radius

Treatment		**Control**			**TRI**	
retinal radius	**1/6**	**3/6**	**5/6**	**1/6**	**3/6**	**5/6**
mice strain			**MGCre-B6**			
RGC survival						
cells/mm²	3275 ± 166	2912 ± 180	1975 ± 41	904 ± 99	891 ± 36	1063 ± 58
Percentage vs. contralateral eye	104 ± 11%	98 ± 13%	116 ± 9%	25 ± 3%	29 ± 1%	63 ± 5%
Mean ± SEM		**106 ± 11%**			**35 ± 2%**	
n=		4			6	
mice strain			**MGCre-FXN**			
RGC survival						
cells/mm²	3258 ± 339	2974 ± 207	2103 ± 139	1358 ± 109*	1416 ± 159*	1098 ± 192
Percentage vs. contralateral eye	96 ± 10%	104 ± 11%	105 ± 12%	54 ± 9%*	54 ± 11%*	60 ± 9%
Mean ± SEM		**102 ± 10%**			**58 ± 6% ***	
n=		4			8	

Table 2: Primer sequences for mRNAs analyzed in the study, together with the expected product length.

Gene name		Primer Seq. (5'->3')	Prod. size (bp)
hFXN human Fratxin	Fw Rev	CCTTGCAGACAAGCCATACA CCACTGGATGGAGAAGATAG	150
Fxn mouse FXN	Fw Rev	CCTGGCCGAGTTCTTTGAAG GCCAGATTTGCTTGTTTGG	152
Antioxidant response			
Gpx1 glutathione peroxidase 1	Fw Rev	GGGACTACACCGAGATGAACGA ACCATTCACTTCGCACTTCTCA	197
Sod1 superoxide dismutase [Cu-Zn]	Fw Rev	GTCCGTCGGCTTCTCGTCT CACAACTGGTTCACCGCTTG	163
Sod2 superoxide dismutase [Mn]	Fw Rev	ATTAACGCGCAGATCATGCA TGTCCCCCACCATTGAACTT	161
Cat catalase	Fw Rev	GCAGATACCTGTGAACTGTC GTAGAATGTCCGCACCTGAG	229
Hmox1 heme oxygenase 1	Fw Rev	GGTGATGGCTTCCTTGTACC AGTGAGGCCCATACCAGAAG	155
Glial response			
Vim Vimentin	Fw Rev	TGAAGGAAGAGATGGCTCGT GGTGTCAACCAGAGGAAGTGA	194
Gfap glial fibrillary acidic protein	Fw Rev	AGAAAGGTTGAATCGCTGGA GCCACTGCCTCGTATTGAGT	176
Glast Glutamate aspartate transporter	Fw Rev	GCCCTCCGACCGTATAAAAT GCCATTCCTGTGACGAGACT	128
Glul glutamine synthetase	Fw Rev	ATCGGGTGTGCGAAGACTTT CGAATGTGGTACTGGTGCCT	181
Neurotrophic factors			
Lif leukemia inhibitory factor	Fw Rev	AATGCCACCTGTGCCATACG CAACTTGGTCTTCTCTGTCCCG	216
Ngf nerve growth factor	Fw Rev	AGCATTCCCTTGACACAG GGTCTACAGTGATGTTGC	99
Bdnf brain-derived neurotrophic factor	Fw Rev	TGGCTGACACTTTTGACCAC CAAAGGCACTTGACTGCTGA	131
Cntf ciliary neurotrophic factor	Fw Rev	ATGACTGAGGCAGAGCGACT AGGCAGAAACTTGGAGCGTA	157
Gdnf glial cell line-derived neurotrophic factor	Fw Rev	TGGGCTATGAAACCAAGGAG CAACATGCCTGGCCTACTTT	142
Inflammatory markers			
Tnf-alpha Tumor necrosis factor alpha	Fw Rev	GTCTACTGAACTTCGGGGTGAT ATGATCTGAGTGTGAGGGTCTG	102
Il1b Interleukin 1 beta	Fw Rev	GAAGAGCCCATCCTCTGTGA TTCATCTCGGAGCCTGTAGTG	96

Housekeeping genes

Hprt hypoxanthine-guanine phosphoribosyltransferase	Fw Rev	TGACACTGGTAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	94
Gapdh Glyceraldehyde 3-phosphate dehydrogenase	Fw Rev	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	123

fw, forward primer; rev, reverse primer

Figure 1

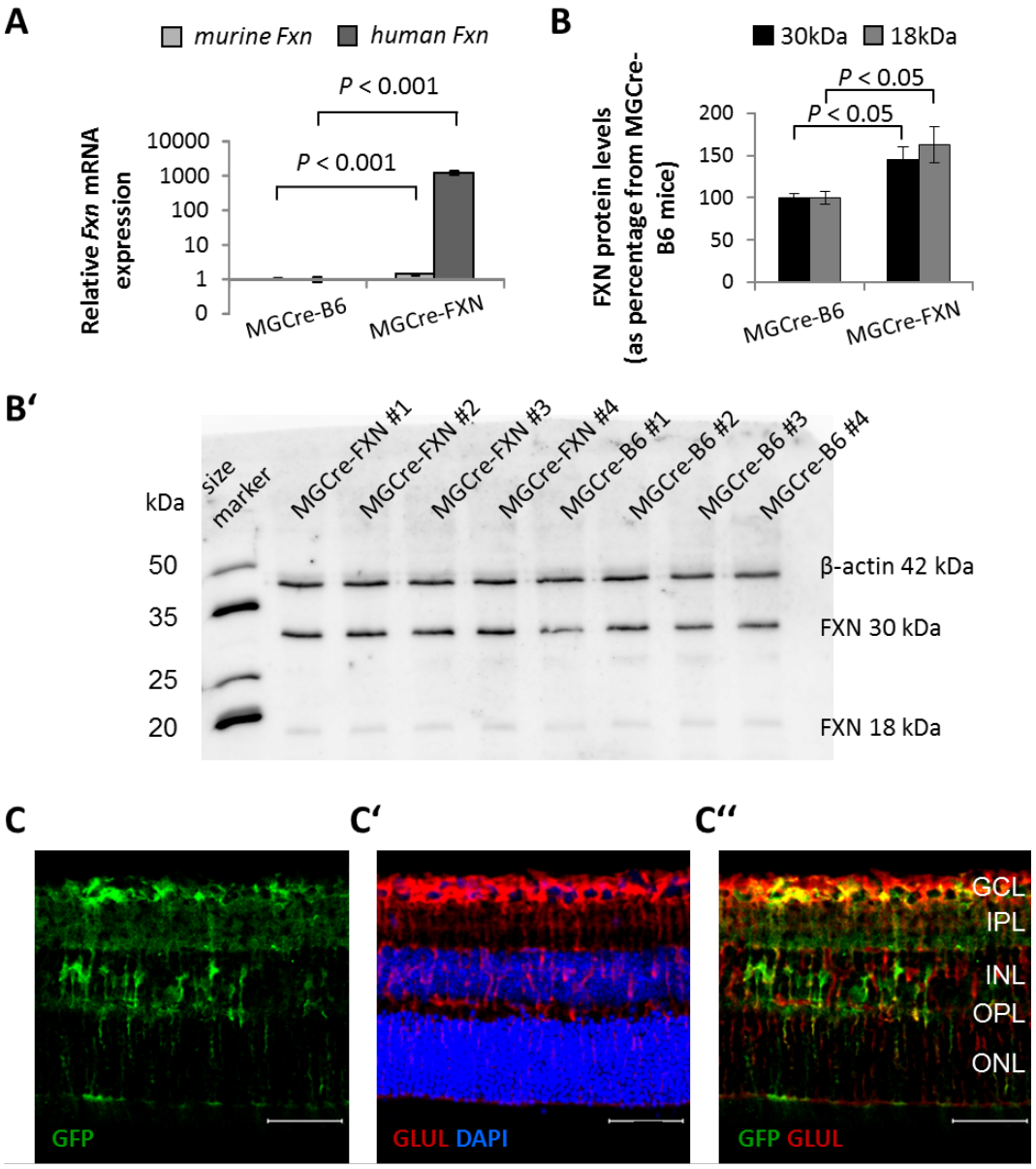

A
murine Fxn
human Fxn
P < 0.001
P < 0.001
Relative Fxn mRNA expression
10000
1000
100
10
1
0
MGCre-B6
MGCre-FXN
B
30kDa
18kDa
P < 0.05
P < 0.05
FXN protein levels (as percentage from MGCre-B6 mice)
200
150
100
50
0
MGCre-B6
MGCre-FXN
B'
kDa
size marker
MGCre-FXN #1
MGCre-FXN #2
MGCre-FXN #3
MGCre-FXN #4
MGCre-B6 #1
MGCre-B6 #2
MGCre-B6 #3
MGCre-B6 #4
50
35
25
20
β-actin 42 kDa
FXN 30 kDa
FXN 18 kDa
C
C'
C''
GFP
GLUL DAPI
GFP GLUL
GCL
IPL
INL
OPL
ONL

Figure 2

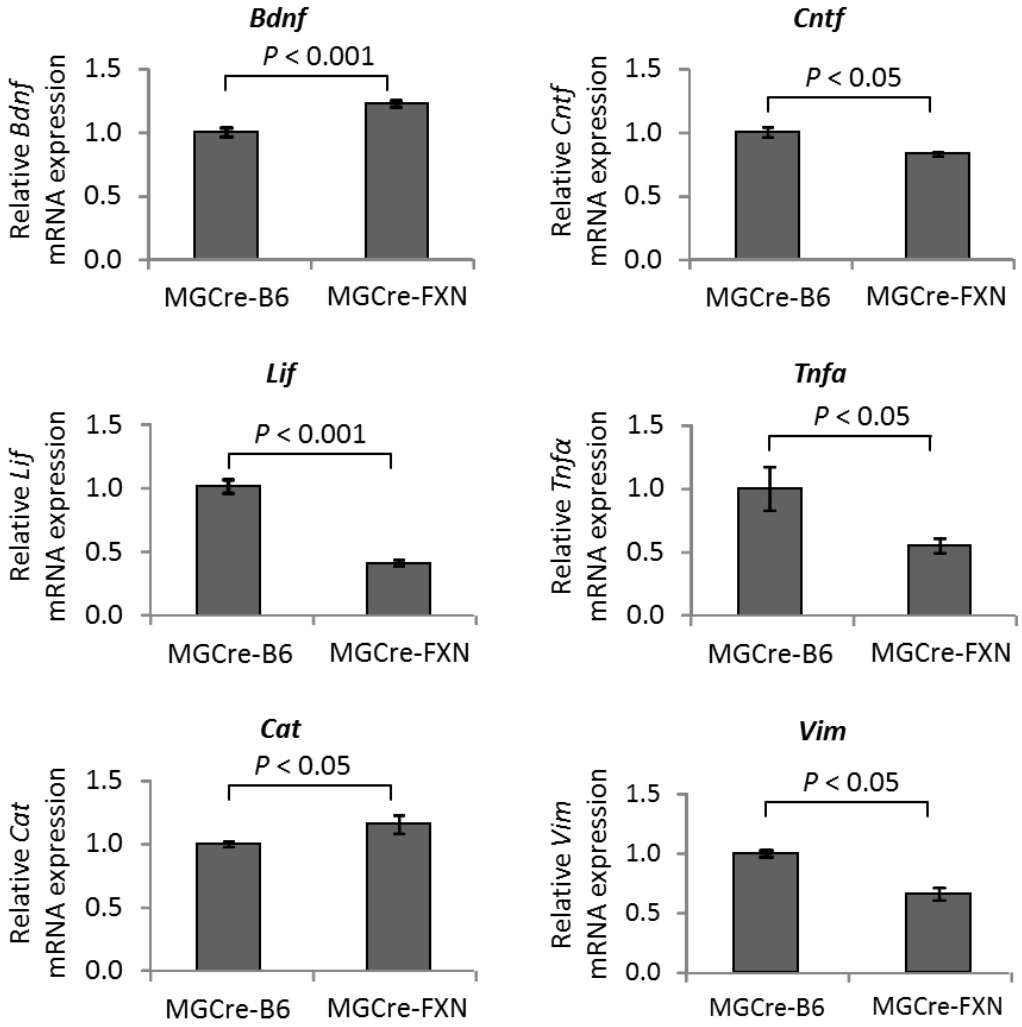

Bdnf
P < 0.001
Relative Bdnf mRNA expression
1.5
1.0
0.5
0.0
MGCre-B6
MGCre-FXN
Cntf
P < 0.05
Relative Cntf mRNA expression
Lif
P < 0.001
Relative Lif mRNA expression
Tnfa
P < 0.05
Relative Tnfα mRNA expression
Cat
P < 0.05
Relative Cat mRNA expression
Vim
P < 0.05
Relative Vim mRNA expression

Figure 3

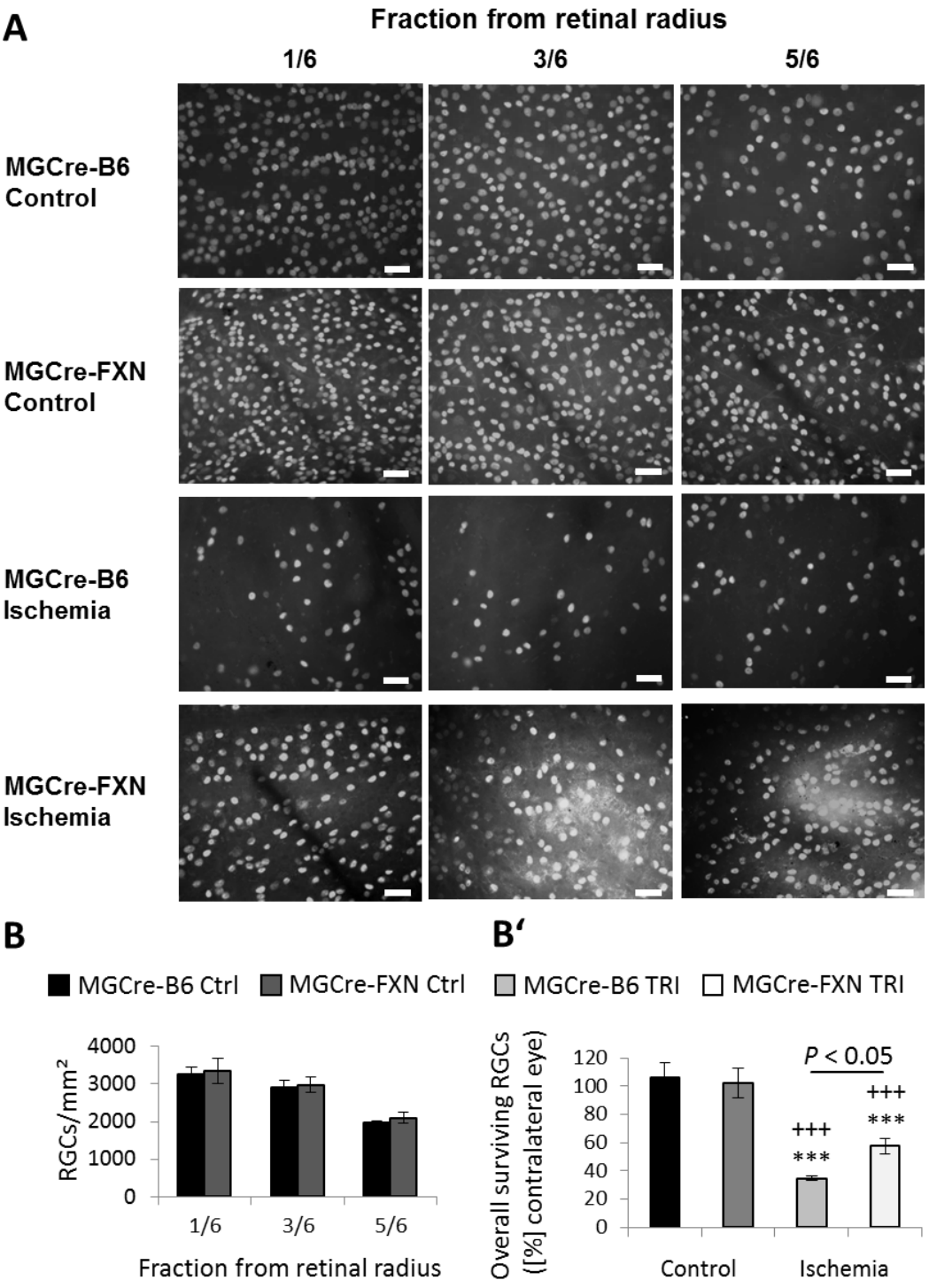

A
Fraction from retinal radius
1/6
3/6
5/6
MGCre-B6 Control
MGCre-FXN Control
MGCre-B6 Ischemia
MGCre-FXN Ischemia
B
MGCre-B6 Ctrl
MGCre-FXN Ctrl
RGCs/mm²
4000
3000
2000
1000
0
1/6
3/6
5/6
Fraction from retinal radius
B'
MGCre-B6 TRI
MGCre-FXN TRI
Overall surviving RGCs ([%] contralateral eye)
120
100
80
60
40
20
0
P < 0.05
+++
***
+++
***
Control
Ischemia

Figure 4

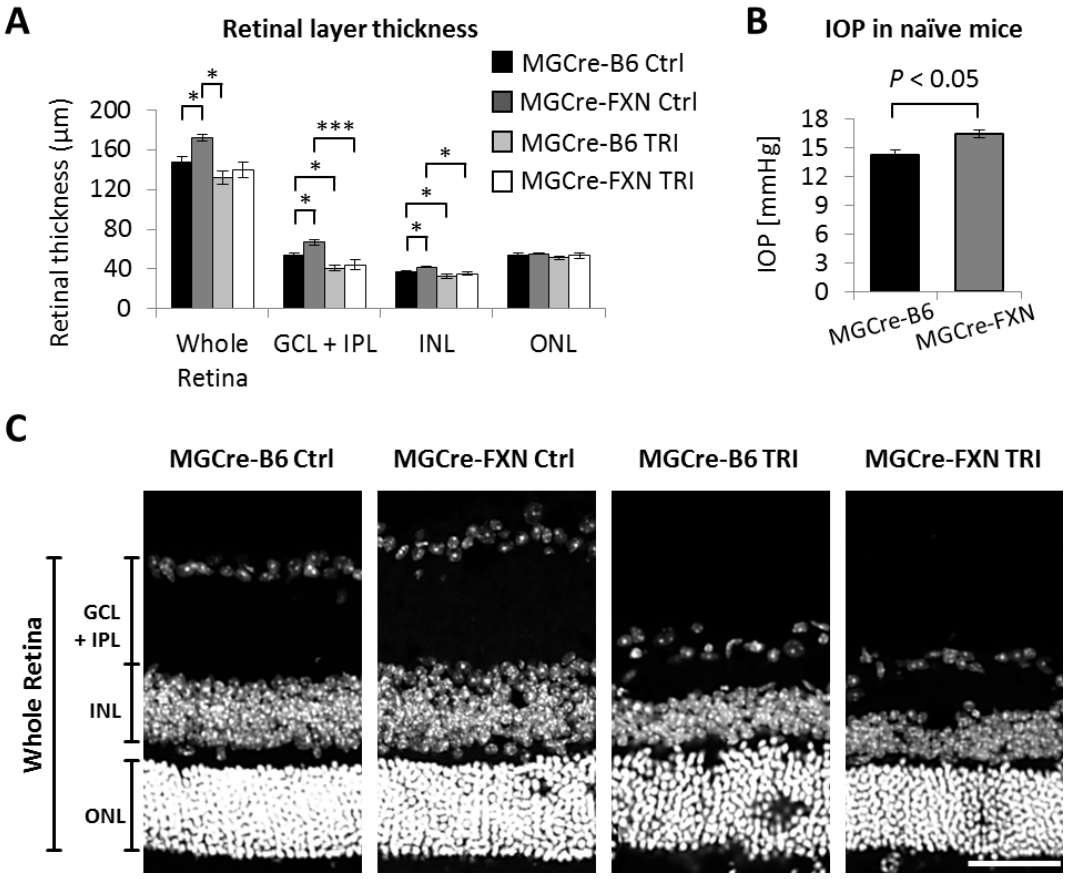

A
Retinal layer thickness
MGCre-B6 Ctrl
MGCre-FXN Ctrl
MGCre-B6 TRI
MGCre-FXN TRI
Retinal thickness (µm)
200
160
120
80
40
0
Whole Retina
GCL + IPL
INL
ONL
B
IOP in naïve mice
P < 0.05
IOP [mmHg]
18
15
12
9
6
3
0
MGCre-B6
MGCre-FXN
C
MGCre-B6 Ctrl
MGCre-FXN Ctrl
MGCre-B6 TRI
MGCre-FXN TRI
Whole Retina
GCL + IPL
INL
ONL

Figure 5

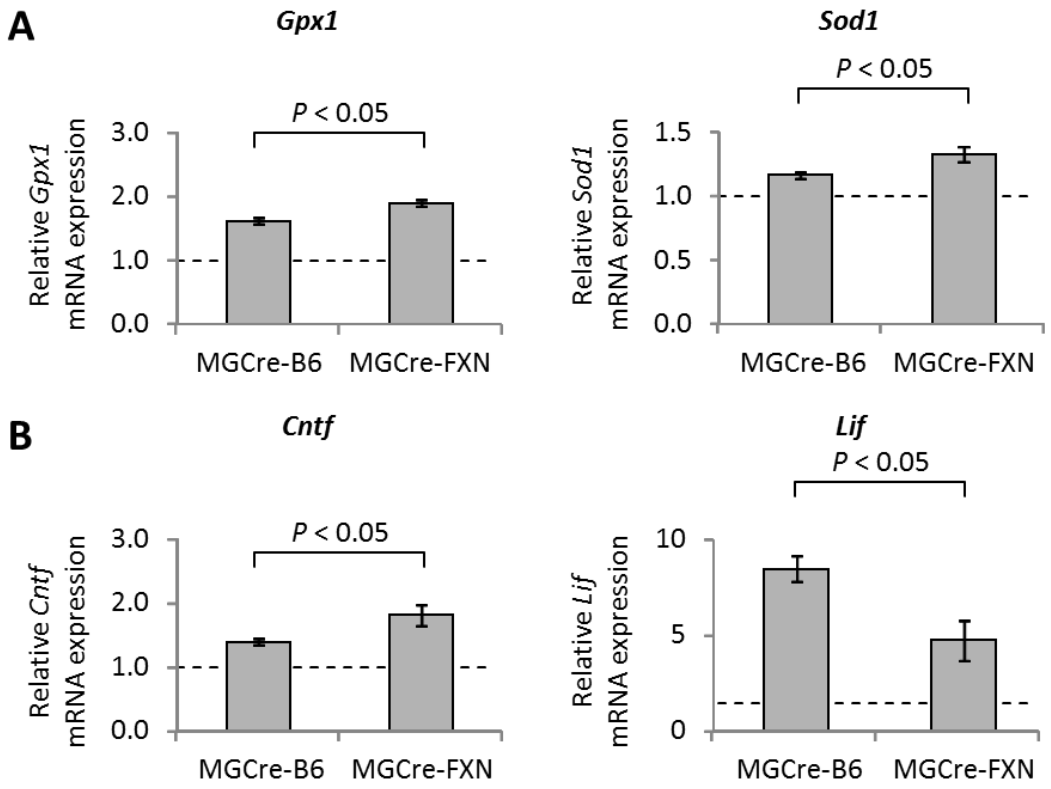

A
Gpx1
P < 0.05
Relative Gpx1 mRNA expression
3.0
2.0
1.0
0.0
MGCre-B6
MGCre-FXN
Sod1
P < 0.05
Relative Sod1 mRNA expression
1.5
1.0
0.5
0.0
MGCre-B6
MGCre-FXN
B
Cntf
P < 0.05
Relative Cntf mRNA expression
3.0
2.0
1.0
0.0
MGCre-B6
MGCre-FXN
Lif
P < 0.05
Relative Lif mRNA expression
10
5
0
MGCre-B6
MGCre-FXN

Figure 6

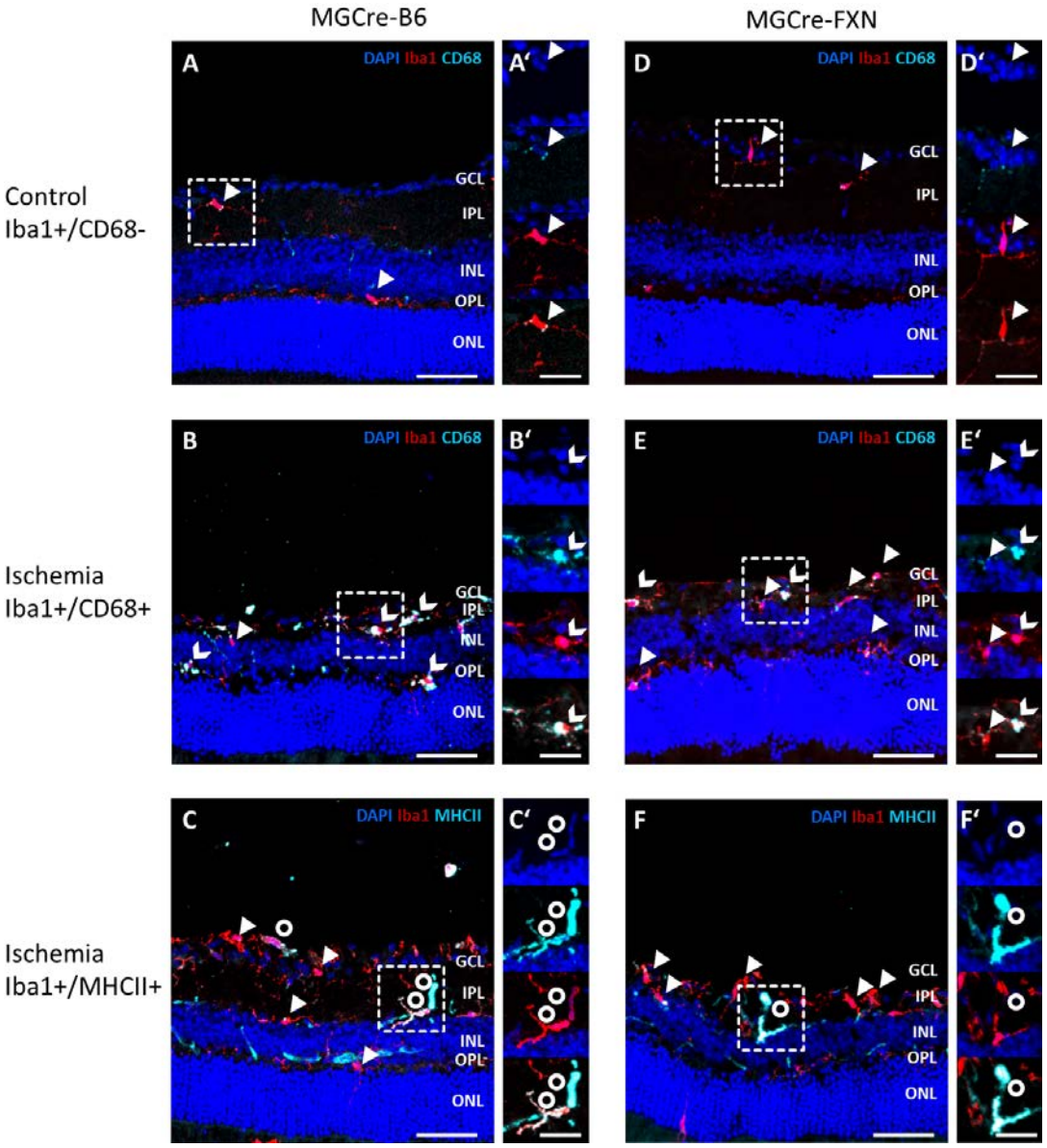
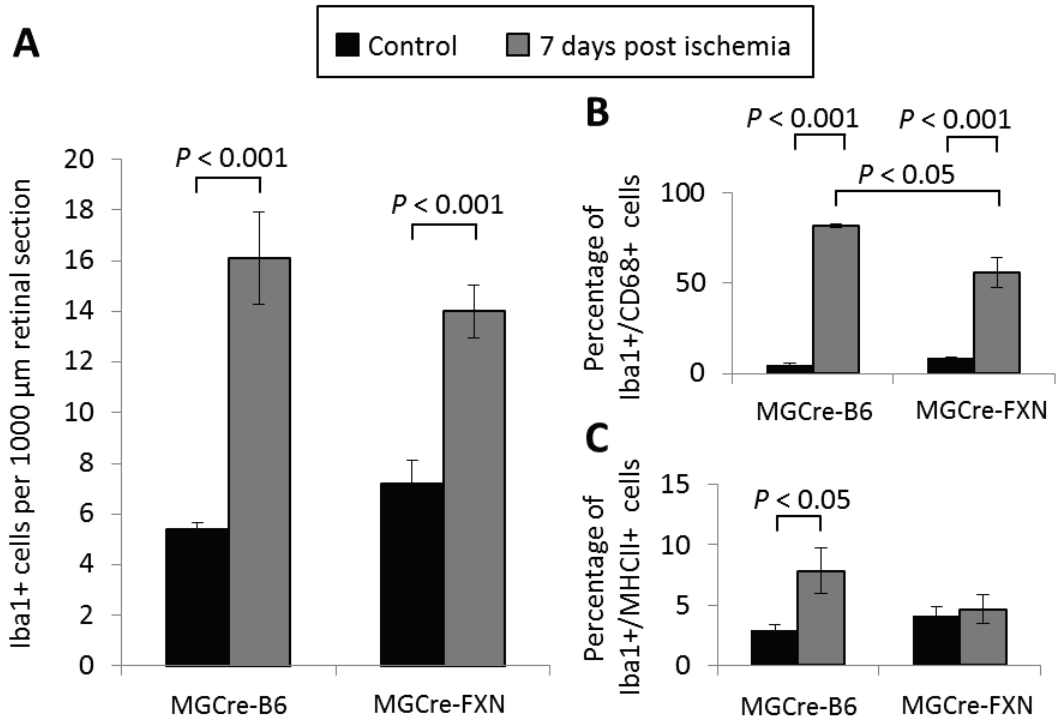

MGCre-B6
MGCre-FXN
Control
Iba1+/CD68-
Ischemia
Iba1+/CD68+
Ischemia
Iba1+/MHCII+
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
DAPI Iba1 CD68
DAPI Iba1 MHCII
GCL
IPL
INL
OPL
ONL

Figure 7


A
Control
7 days post ischemia
Iba1+ cells per 1000 µm retinal section
20
18
16
14
12
10
8
6
4
2
0
P < 0.001
P < 0.001
MGCre-B6
MGCre-FXN
B
P < 0.001
P < 0.001
P < 0.05
Percentage of Iba1+/CD68+ cells
100
50
0
MGCre-B6
MGCre-FXN
C
Percentage of Iba1+/MHCII+ cells
15
10
5
0
P < 0.05
MGCre-B6
MGCre-FXN

Frataxin overexpression in Müller cells protects retinal ganglion cells in a mouse model of acute glaucoma in vivo.

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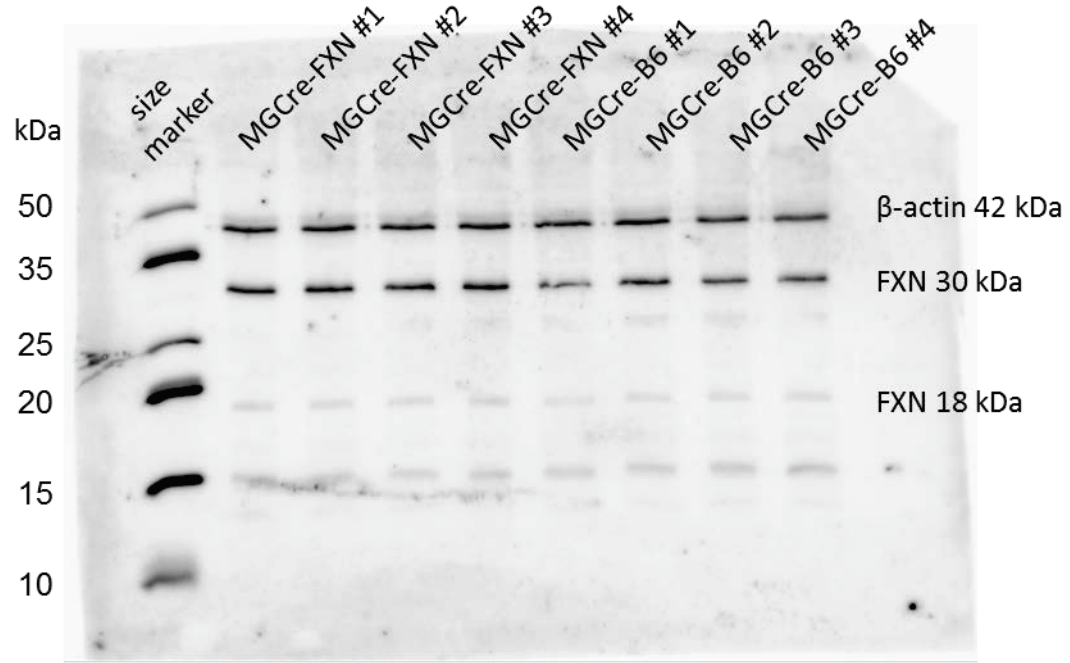
Supplementary information

Supplementary Figure 1: Representative Western blot showing FXN precursor and mature bands for FXN in MGCre-B6 and MGCre-FXN mice, full-length blot.



5 Closing discussion

Most retinal pathologies, including diabetic retinopathy, macular degeneration, retinal detachment, glaucoma and ischemia, result in the activation of Müller cells, the most abundant retinal glial cell type (Bringmann *et al.* 2009). The disease or lesion-induced response of Müller cells includes the buffering of elevated potassium levels and uptake of excess glutamate, which would otherwise be neurotoxic (Lucas and Newhouse 1957; Napper *et al.* 1999; Bringmann *et al.* 2009). Moreover, Müller cells release neurotrophic factors, growth factors, and cytokines to protect photoreceptors and neurons from cell death (Harada *et al.* 2000; Fontaine *et al.* 2002; Fu *et al.* 2015; Garcia *et al.* 2014). Conversely, Müller cell activation can also have detrimental effects, for instance by VEGF-induced leakage and neovascularization (Croll *et al.* 2004; Yasuhara *et al.* 2004; Kilic *et al.* 2006; Pierce *et al.* 1995; Tolentino *et al.* 2002) or impaired neuronal recovery and axonal regrowth by glial-secreted extracellular matrix substrates (Shen *et al.* 2008; Moreau-Fauvarque *et al.* 2003). Moreover, Müller cells mediate cytotoxicity by the release of soluble factors such as the proinflammatory cytokine TNF-α which activates TNF-R1 and triggers apoptosis (Cotinet *et al.* 1997; Tezel *et al.* 2001).

The precise timing for the transition between the supportive and detrimental responses of Müller cells to lesion, as well as the underlying mechanisms is poorly understood. The critical role of Müller glia in regulating neuronal homeostasis, but also their transition to a detrimental gliotic response after injury or in disease, converts them to a potential therapeutic target for the treatment of neurodegenerative retinal diseases.

This study aimed to determine whether modulation of the Müller cell gliotic response after injury constitutes a feasible target to reduce neuronal damage after an ischemic injury to the retina by answering two questions: i) what is the time course for the development of the Müller cells neuroprotective and detrimental responses after an acute ischemic injury, and ii) what is the effect of selective FXN overexpression, and its associated antioxidative activity in Müller cells on neuronal survival after ischemia.

5.1 The development of neuroprotective and detrimental responses of Müller cells after lesion is time-dependent

Worldwide, glial cells are currently the focus of research in many labs, and increasing evidence supports their critical role in brain function during health and disease. Approaches

to modulate glial activity and to reduce neuronal damage after injury have recently been evaluated. In particular, several studies have focused on the regulation of the gliotic activity of Müller cells. For instance, inhibition of reactive gliosis, by intravitreal injection of the glial toxin neurostatin, prevents kainic acid-induced apoptotic death of retinal neurons and provides substantial neuroprotection (Ganesh and Chintala 2011). Inhibition of intermediate filament polymerization with withaferin A, a blocker of type III intermediate filament dynamics, prevented a p38 MAPK-dependent secretion of TNF-α, resulting in reduced Müller cell gliosis and neuronal apoptosis (Livne-Bar *et al.* 2016). FC has also been used to inhibit glial cells and to study the impact of glial metabolic impairment on neuronal cells *in vitro* and *in vivo*. In contrast to the aforementioned studies, FC-mediated inhibition of glial cells was detrimental for neurons. Inhibition of astrocytic mitochondria with 0.25 mM FC, prior to glutamate treatment, increased the vulnerability of co-cultured neurons to glutamate toxicity (Volobouéva *et al.* 2007). In addition, inhibition of glial cell activity with 1.0 mM FC, 10 minutes before middle cerebral artery occlusion (MCAO), caused significant cell damage in rats (Hosoi *et al.* 2006). These findings demonstrated that inhibition of glial metabolism increases neuronal vulnerability to a short-term transient ischemia. FC administered after MCAO not only inhibited reactive astrocytes, but also suppressed neurovascular markers and worsened the neurological outcome (Hayakawa *et al.* 2010; Hayakawa *et al.* 2009). In this study, FC was given once every 2 days starting 5 days after ischemia which showed that inhibition of glial cells at late reperfusion also increases neuronal susceptibility to ischemia.

In addition to the aforementioned studies, the impact of glial cell inhibition after lesion has been previously evaluated, for instance, by the post-conditioning paradigm. In the retina, post-conditioning has been induced by re-increasing the IOP immediately or as late as 24 hours after ischemia (Dreixler *et al.* 2010; Fernandez *et al.* 2009a). Both immediate and delayed post-conditioning were found to reduce glial cell reactivity concomitant with a robust functional recovery and increased survival of RGCs after ischemia (Fernandez *et al.* 2009a; Schallner *et al.* 2012; Fernandez *et al.* 2009b; Dreixler *et al.* 2011). These findings argue against a specific timing for the transition from neuroprotective to detrimental gliosis. However, post-conditioning is not a cell-specific mechanism and affects both the glial- and neuronal response to ischemia.

The question on the effect of a specific down-regulation of the gliotic Müller cell response on neuronal survival in the acute and sub-chronic phase after lesion remained unanswered. Addressing this question is highly relevant to determine a putative time window for

therapeutic interventions aimed at reducing neuronal damage after lesion. In this study, in order to evaluate the time-course for the development of neuroprotective and detrimental responses in Müller cells, the cellular metabolism was transiently inhibited with FC, as previously described (Virgili *et al.* 1991). This was done at different time points before and during the reperfusion phase after ischemia. In agreement with previous findings (Volobouéva *et al.* 2007; Hosoi *et al.* 2006), a transient metabolic inhibition in Müller cells at lesion onset was not neuroprotective, but rather increased neuronal cell death. Therefore, the gliotic response of Müller cells during and early after ischemia supports neuronal survival. In fact, metabolic inhibition did not affect RGCs survival for up to 10 hours after ischemia. During this time, Müller cells seem not to influence the degeneration of RGCs. However, Müller cell inhibition starting 12-18 after lesion leads to increased neuronal survival. Hence, the Müller cell response to lesion seems to become detrimental during this time frame.

In conclusion, the Müller cell gliotic response is neuroprotective at the onset of a retinal ischemia/reperfusion injury, but progresses to a more detrimental response 12 to 18 hours after reperfusion onset. This transition can be modulated using a specific glial metabolic inhibitor. Findings indicate a time window of at least 6 hours starting 12 hours after reperfusion which would allow for therapeutic interventions aimed to reduce detrimental gliotic effects and improve neuronal survival after an acute ischemic injury. Future studies will determine whether these positive effects are long-lasting or only of short duration.

5.2 Frataxin overexpression improves the intrinsic antioxidative capacity in the retina

One hallmark of retinal ischemia/reperfusion injury is the generation of excessive ROS during reperfusion. Glial cells react to increased oxidative stress by releasing anti-oxidative factors (Schütte and Werner 1998; Bringmann and Reichenbach 2001; García and Vecino 2003; Bringmann *et al.* 2006). In this study, protocols to enhance the anti-oxidative response in the retina and, specifically in Müller cells, were established in order to improve the glial neuroprotective activity and to reduce detrimental effects of the gliotic response during and after lesion. This was achieved by generating transgenic mice overexpressing a copy of the human mitochondrial enzyme FXN ubiquitously in the retina or specifically in retinal Müller cells. We hypothesized that FXN overexpression would reduce oxidative stress after ischemia resulting in increased neuronal survival. Results show that an increased expression of the endogenous mouse FXN is part of the intrinsic retinal response

to ischemia. FXN overexpression in transgenic mice resulted in improved neuronal survival. Furthermore, ubiquitous overexpression of human FXN resulted in elevated levels of enzymes involved in oxidative stress response, namely glutathione peroxidase 1 (*Gpx1*), superoxide dismutase 2 (*Sod2*) and catalase, all of which are well known for their anti-oxidative properties. Importantly, mRNA expression levels of heme oxygenase-1 (*Hmox1*) and hypoxia inducible factor 2 alpha (*Hif2α*), were also increased after ischemia in FXN overexpressing mice compared to non-transgenic controls. Both factors are known to be selectively expressed by Müller cells (Arai-Gaun *et al.* 2004; Mowat *et al.* 2010). This finding indicated that Müller cells might play a critical role in the FXN-mediated neuroprotective response following ischemia. To test for the role of Müller cells in the FXN-mediated response to ischemia and the feasibility to selectively modulate their activity towards a more neuroprotective phenotype, a second mouse model which specifically overexpresses FXN in Müller cells was generated. FXN overexpression increased RGC survival to a similar extent as in mice ubiquitously overexpressing FXN in the retina. Also in this paradigm, anti-oxidant enzymes were significantly involved in the response to the ischemic lesion. In particular, *Gpx1* was significantly increased to the same extent in both FXN overexpressing mice strains after lesion. Findings from this study are in agreement with previous studies showing that FXN overexpression in murine 3T3L1 cells activates glutathione peroxidase and increases levels of thiols, thereby exerting anti-oxidative effects (Shoichet *et al.* 2002). In addition, FXN overexpression in transgenic flies, renders them resistant to toxicity induced by iron, paraquat, and hydrogen peroxide, suggesting that FXN may protect cells against oxidative stress (Runko *et al.* 2008). Furthermore, delivery of the fusion proteins Tat-FXN and PEP-1-FXN suppressed neuronal cell death in a transient forebrain ischemia model. PEP-1-FXN enhanced cell proliferation and neuroblast differentiation by reducing lipid peroxidation and Tat-FXN upregulated the expression of superoxide dismutase and consequently inhibited the generation of 4-hydroxynonenal, which is produced by lipid peroxidation (Kim *et al.* 2010; Kim *et al.* 2011).

It is important to note that for our study we used a constitutive and a conditional mouse model overexpressing FXN. In both of these models FXN was already overexpressed prior to the lesion. In the constitutive mouse model (Deleter mouse), Cre-recombinase was expressed under the transcriptional control of a human cytomegalovirus minimal promoter in all tissues (Schwenk *et al.* 1995). Expression of Cre-recombinase in Müller cells was controlled by the promoter of the human vitelliform macular dystrophy (VMD)-2 (Ueki *et al.* 2009; Zhu *et al.* 2010; Zhou *et al.* 2014). This mouse model was the only available model for a Müller cell-specific Cre expression; however, the VMD-2-promoter is only active in Müller

cells during early embryonic development (Zhu *et al.* 2010). Hence, overexpression of FXN was induced starting as early as embryonic day 15. This means that for both transgenic approaches, FXN was already overexpressed during retinal development. Interestingly, FXN overexpression during development has been described to have deleterious effects in *Drosophila* (Navarro *et al.* 2011), but not in mice (Miranda *et al.* 2004; Schulz *et al.* 2010). Although no obvious alterations were found in other mouse strains overexpressing FXN, an increased number of damaged lenses comparable to those found in animals with cataract (Varma *et al.* 1984) was noticed in this study. Hence, these mice were excluded from the studies. In addition, partially altered retinal structures with expanded retinal layers including GCL/IPL and INL, which were thickened by approximately 20%, were observed. Furthermore, changes in mRNA expression levels for *Tnfα*, *Bdnf*, *Cntf*, *Lif*, catalase and vimentin, and slight, but significant higher IOP were found in naïve transgenic mice compared to non-transgenic mice. Different IOP levels ranging from 11.1 ± 0.5 mmHg to 19.3 ± 0.3 mmHg have been described in different mouse strains (Savinova *et al.* 2001). However, these different IOPs did not result in morphological changes or development of glaucoma. Nevertheless, it cannot be ruled out that early additional copies of FXN might influence retinal development and Müller cell functions before lesion, resulting in an induction of long-lasting retinal ischemic tolerance. This is comparable to preconditioning, which is also neuroprotective and has been shown to occur after treatment with the iron chelator deferroxamine (Zhu *et al.* 2008). Whether a post-lesion-induced increase of FXN would lead to a similar neuroprotective effect after an ischemic injury remains an open question.

In conclusion, FXN overexpression improves the intrinsic response to oxidative stress which results in increased neuronal survival. This supports the notion that improving the Müller cell response to oxidative stress during retinal ischemia is a feasible approach to achieve neuroprotection and to overcome detrimental gliotic effects. In addition, results indicate that in spite of improved neuronal survival after injury, genetic interventions like the one described in this study might also have unpredictable side effects with undefined morphological and functional consequences, which have to be carefully evaluated.

5.3 Signaling pathways involved in cell death and neuroprotection in this study

At present there are no effective treatments for most retinal degenerative diseases; however, a variety of potential approaches are being evaluated including preventive

strategies that aim to counteract the underlying disease mechanisms (e.g. pharmacological or genetic interventions to alter specific cellular pathways and genes). Other strategies focus on cell death prevention by treatment with neurotrophic factors, or cell replacement therapies by transplantation. Development of effective therapeutic treatments for neurodegenerative diseases requires analysis of temporal changes in the cellular responses with the affected gene networks and signaling pathways.

Importantly, retinal neurons are not compromised simultaneously, but die at different rates after ischemia. Cell death can be executed by at least two well-established mechanisms, necrosis and apoptosis. Necrosis starts within the first four hours after acute retinal ischemia but is rarely detectable after 1 day of reperfusion (Joo *et al.* 1999). Apoptosis of neuronal cells starts 6 to 12 hours after ischemia and peaks at 24 hours of reperfusion (Katai and Yoshimura 1999). In addition, cell death can occur following an alternative programmed mechanism, known as necroptosis (Rosenbaum *et al.* 2010; Dvoriantchikova *et al.* 2014). Necroptosis seems to take place within the first 12 to 24 hours after injury (Gao *et al.* 2014). In addition to these two waves of neuronal cell death, our study provides evidence that also the gliotic response after retinal ischemia is comprised of two main time-dependent components, a first early neuroprotective phase followed by a late detrimental second phase. However, our results support the notion that cells dying by necrosis immediately after lesion cannot be effectively rescued. In contrast, cells dying by apoptosis or necroptosis can be rescued by means of different approaches (Chinskey *et al.* 2014)., including those described in this study.

During lesion and reperfusion onset, Müller cells seem to be very important for neuronal survival. In this initial phase, differentially expressed genes are related to changes in metabolism (Andreeva *et al.* 2014). Several studies show that metabolic support via injecting dextrose or glucose into the eye vitreous during ischemia is neuroprotective (Büchi *et al.* 1991; Romano *et al.* 1993; Romano *et al.* 1998; Casson *et al.* 2004). In support of this, we show that reduced Müller cell activity during this phase impairs RGCs survival after lesion. Delivery of neurotrophic factors during or immediately after injury also sustains ganglion cell survival (Wong *et al.* 2014). Importantly, targeting the sub-chronic phase after ischemic injury, which is characterized by enhanced inflammatory and immune responses as well as highly regulated cell death related genes (Andreeva *et al.* 2014; Produit-Zengaffinen *et al.* 2009) is also effective in neuroprotection and clinically relevant. Delayed administration of neurotrophic factors in retinal injury was shown to improve neuronal survival (Kyhn *et al.* 2009; Lambiase *et al.* 2009; Xiao and Zhang 2010; Checa-Casalengua *et*

al. 2011; Yang *et al.* 2011; Colafrancesco *et al.* 2011). In agreement with this, inhibition of Müller cell response to ischemia by FC increased and prolonged expression of endogenous neurotrophic factors including *Bdnf*, *Gdnf* and *Ngf*. In general, NGF and BDNF control neuronal survival via two types of receptors: the tropomyosin receptor kinase (Trk) family of high-affinity tyrosine kinase receptors which is associated with neuronal survival, and the low-affinity $p75^{NTR}$ receptor which is implicated in neuronal apoptosis and mainly expressed in Müller cells. In addition, the GDNF receptors GFRα1 and RET have also been localized to Müller cells (reviewed by Kimura *et al.* 2016; Bringmann *et al.* 2009). One might speculate that the diminished metabolism of Müller cells after FC treatment leads to an altered response of these cells to neurotrophic factors, thereby influencing neurotrophic factor expression. In support of this, GDNF positively regulates its own expression, leading to a long-lasting activation of the GDNF signaling pathway in neuronal cell cultures (He and Ron 2006), whereas a microRNA-BDNF negative feedback signaling loop has been described in the brain (Keifer *et al.* 2015). An autocrine loop involving TNF-α contributes to the production of NGF and GDNF in astrocytes (Kuno *et al.* 2006). Interestingly TNF-α is increased in FC treated mice. Therefore, inhibition of Müller cells might directly lead to a prolonged neuron-supportive signaling of these neurotrophic factors. However, further research is needed to support this notion. Interestingly, supporting Müller cells by FXN overexpression also leads to increased expression of neurotrophic factors. In particular *Cntf,* which is typically expressed by Müller cells, is higher expressed (Ji *et al.* 2004; Honjo *et al.* 2000). This evidence argues for the involvement of a different neuroprotective pathway in this Müller cell response compared to FC treated Müller cells. Nevertheless, all of these factors are well described for reducing cellular apoptosis and the expression of cell death related genes (Kimura *et al.* 2016).

The two approaches evaluated in this study led to increased expression of antioxidant enzymes. Oxidative stress is, as previously mentioned, one of the main triggers for RGC death in retinal ischemia/reperfusion injury (Osborne *et al.* 2004; Williams 2008) and therefore, enhanced antioxidant enzyme expression directly correlates with increased neuronal survival observed here (Arai-Gaun *et al.* 2004; Liu *et al.* 2012b). Furthermore, oxidative stress is linked to inflammation in glaucoma (Tezel 2006; Pinazo-Durán *et al.* 2013). Importantly, microglia reactivity was decreased in FXN mice. Reduced microglia reactivity might result from a better antioxidant capacity of the Müller cells and improved function of these cells. The possible mechanism linking increased Müller cell function and decreased microglia reactivity will be addressed in future studies.

In conclusion, it was shown that the transition from a neuroprotective to detrimental gliotic response in Müller cells seems to be timed with changes in the inflammatory and immune responses as well as the regulation of cell death related genes. Impairing the Müller cell metabolism during this transition helped to reduce detrimental effects by increasing and prolonging the expression of endogenous neurotrophic factors and antioxidant enzymes. Support of Müller cells by increasing their oxidative stress response was also associated with increased levels of neuroprotective factors and decreased microglial reactivity. However, the sources of the neuroprotective or regulatory factors are not known. Some factors are produced by Müller cells, but further studies are required to identify the cell types responsible for the increased expression of these factors.

5.4 Combined neuroprotective strategies: alternatives to improve neuronal survival after lesions?

One interesting observation in this study was the fact that, independently of the approach used (FC or FXN) the degree of survival of the neuronal cell population did not increase over 60-70% (figure 2). Similar survival rates were also described by other researchers using the same lesion model. Zhu *et al.* (2002) obtained a survival rate of 65% after preconditioning. Delivery of Nec1, an inhibitor of necroptosis, one hour prior to an ischemic injury, rescued 66% of RGCs (Dvoriantchikova *et al.* 2014). Ueda *et al.* (2004) delivered nefiracetam, which activates voltage-dependent N-type and L-type calcium channels, 30 min before or 24 hours after lesion onset and also obtained a neuronal survival rate of 65%. Hence, different approaches for neuroprotection lead to similar levels of neuronal survival after acute retinal ischemia. These findings point to a limit in the number of cells that can be saved following a retinal insult, independently of the strategy used. One possible reason for this is that some cells are damaged beyond recovery and cannot be rescued, as already discussed. Another reason might be that most approaches rely on the use of pharmacological and genetic strategies which target one specific pathway in a single cell population, mostly neuronal.

It is conceivable that this apparent limit might be overcome by using a combined approach targeting one or more mechanisms in multiple cell types. Such an approach has already been evaluated for focal cerebral ischemia by combining the positive effects of therapeutic hypothermia with drugs interrupting one or more events of the ischemic cascade (reviewed by Goossens and Hachimi-Idrissi, 2014). The use of combined therapies might also serve to extend the time window for further delivery of additional neuroprotective agents (Qian *et al.* 2016; Aronowski *et al.* 1996; Fu *et al.* 2009).

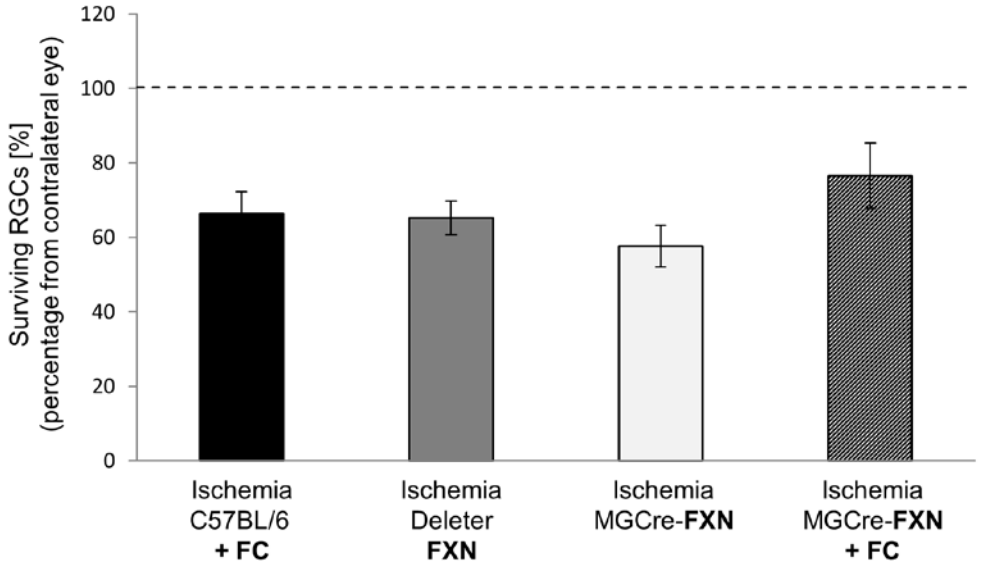


Figure 2: Exemplary graph for the analysis of surviving RGCs 7 to 14 days after acute retinal ischemia/reperfusion injury in mice eye. Numbers of RGCs are expressed as percentages from contralateral non-ischemic eyes. Cell counts were determined 7 days after ischemia in retinae treated with FC and 14 days post lesion in ubiquitous FXN overexpressing (Deleter FXN) and Müller cells-specific FXN overexpressing mice (Vmd2-cre-FXN). Bars represent the mean ± SEM, dashed line represents 100 % of the naïve control eyes.

In order to test this concept, we used a combined approach and delivered FC into FXN overexpressing mice. It was expected that this combination would further increase the neuronal survival after acute ischemia. However, this combined approach did not result in a higher level of RGCs survival (figure 2). It is possible that these two effects do no act synergistically. This is in agreement with other studies using a combination of pre- and post-conditioning, which also did not result in improved neuronal survival compared to either pre- or post-conditioning anlone (Dreixler *et al.* 2010).

As already mentioned, most studies focus mainly on neuronal mechanisms of injury. Recently, a new focus has been set on brain astrocytes i.e. for the treatment of stroke (Barreto *et al.* 2011; Liu and Chopp 2016). In our study, the therapeutic value of targeting Müller cells for the treatment of retinal ischemia was assessed. We show that selective modulation of the function of Müller cells was sufficient to achieve significant neuroprotection after retinal ischemia. In addition, we found that this strategy also led to significant changes in another non-neuronal cell type, the microglia. It is well known that besides gliotic alterations of Müller cells, microglia cells also become reactive in glaucoma (Inman and Horner 2007; Bosco *et al.* 2011; McKinnon *et al.* 2009). Furthermore, a negative

association between the number of surviving RGCs and the extent of microgliosis has already been described (Liu *et al.* 2012a). In the study presented here, decreased microglia reactivity was observed after FXN overexpression. Additionally, also in an MCAO model, microglia are most likely the key player for a worse neurological outcome after late FC administration (Hayakawa *et al.* 2010; Hayakawa *et al.* 2009). Müller glia and microglia communicate in a bi-directional manner by influencing the secretion of neurotrophic and inflammatory factors (Wang *et al.* 2011). Furthermore, microglia can also interact with neurons by secreting growth factors (Vecino *et al.* 2016) and vice versa. Glia cells are affected by factors secreted from healthy or damaged neurons including fractalkine, which suppresses microglia (Cardona *et al.* 2006), or glutamate, which activates glia cells. These findings, which highlight critical interactions between different cell types, strongly support the putative therapeutic value of a combined strategy aimed to target multiple cell populations to increase neuronal survival after lesion or during disease. This issue deserves further research. Morover, later time points after ischemia and FC treatment should be evaluated to clarifiy whether the observed surviving RGCs are only stalled in their progression to neuronal death or whether it represents a true protection. In addition, it would be worthwhile to investigate the effect of the manipulation of Müller cells in slow retinal degenerative disease models such as glaucoma or diabetic retinopathy.

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7 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Otto W. Witte und Dr. Christian Schmeer,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum	Unterschrift des Verfassers

8 Angaben zum Eigenanteil

Increased frataxin levels protect retinal ganglion cells after acute ischemia/reperfusion in the mouse retina in vivo, Rowena Schultz, Otto W. Witte, Christian Schmeer, published in the journal **Investigative Ophthalmology and Visual Science**, 2016, volume 57(10), pp.4115–4124.

Diplom-Biochemikerin Rowena Schultz: Planung und Durchführung der experimentellen Arbeiten, Methodenetablierung (Western Blot für FXN, qPCR für *hFXN* und *mFxn* sowie antioxidative Enzyme), Datenerfassung und statistische Berechnungen, Erstellung eines Großteils des Rohmanuskripts und aller Abbildungen, Überarbeitung der Manuskriptendversion.

Prof. Dr. med. Otto W. Witte: Bereitstellung der Arbeitsmöglichkeit, der Materialen und Geräte, fachliche Diskussionen und Mitwirkung an der Konzepterstellung, Unterstützung bei der Erstellung der Manuskriptendversion.

Ph.D. Christian Schmeer: Vergabe des Themengebietes und Projektplanung, fachliche Einweisung und Unterstützung bei den experimentellen Arbeiten (Induktion einer retinale Ischämie, Gewebeaufbereitung), fachlich-kritische Diskussionen, Unterstützung bei der Erstellung des Rohmanuskriptes und der Manuskriptendversion.

Transient inhibition of Müller cells by fluorocitrate increases neuronal survival after acute retinal ischemia in vivo, Rowena Schultz, Twinkle Vohra, Julia Lindner, Otto W. Witte, Christian Schmeer, under revision at the **Journal of Neurochemistry** (submitted on September 29, 2017)**.**

Diplom-Biochemikerin Rowena Schultz: Planung und Durchführung der experimentellen Arbeiten für die biochemischen Untersuchungen, retinale Ischämie und qPCR, Methodenetablierung (biochemische Untersuchungen, qPCR für Wachstumsfaktoren, Zellkultur), Datenerfassung und statistische Berechnungen, Erstellung des Rohmanuskripts und aller Abbildungen, Überarbeitung der Manuskriptendversion.

Master of Science Twinkle Vohra: Planung und Durchführung der experimentellen Arbeiten für die biochemischen Untersuchungen, Methodenetablierung (FC-Injektion, biochemische Untersuchungen), Mikroskopie (Etablierung der Gewebefärbung und Auswertung), Datenerfassung und statistische Berechnungen, Überarbeitung der Manuskriptendversion.

Master of Science Julia Lindner: Methodenetablierung und Assistenz bei den experimentellen Arbeiten (Zellkultur).

Prof. Dr. med. Otto W. Witte: Bereitstellung der Arbeitsmöglichkeit, der Materialen und Geräte, fachliche Diskussionen und Mitwirkung an der Konzepterstellung, Unterstützung bei der Erstellung der Manuskriptendversion.

Ph.D. Christian Schmeer: Vergabe des Themengebietes und Projektplanung, fachliche Einweisung und Unterstützung bei den experimentellen Arbeiten (Induktion einer retinale

Ischämie, Gewebeaufbereitung), fachlich-kritische Diskussionen, Unterstützung bei der Erstellung der Manuskriptendversion.

Frataxin overexpression in Müller cells protects retinal ganglion cells in a mouse model of acute glaucoma in vivo, Rowena Schultz, Melanie Krug, Michel Precht, Stefanie G. Wohl, Otto W. Witte, Christian Schmeer, has been submitted to on November 17, 2017. published in the journal **Scientific Reports**, 2018, volume 8(1), pp.4846.

Diplom-Biochemikerin Rowena Schultz: Planung und Durchführung der experimentellen Arbeiten, Datenerfassung und statistische Berechnungen, Erstellung des Rohmanuskripts und aller Abbildungen, Überarbeitung der Manuskriptendversion.

Master of Science Melanie Krug: Assistenz bei den experimentellen Arbeiten und der Datenerfassung (Mikroskopie).

Master of Science Michel Precht: Assistenz bei den experimentellen Arbeiten und der Datenerfassung (Mikroskopie).

Dr. rer. nat. Stefanie G. Wohl: Assistenz bei den experimentellen Arbeiten und der Datenerfassung (Mikroskopie), fachlich-kritische Diskussionen, Unterstützung bei der Erstellung der Manuskriptendversion.

Prof. Dr. med. Otto W. Witte: Bereitstellung der Arbeitsmöglichkeit, der Materialen und Geräte, fachliche Diskussionen und Mitwirkung an der Konzepterstellung, Unterstützung bei der Erstellung der Manuskriptendversion.

Ph.D. Christian Schmeer: Vergabe des Themengebietes und Projektplanung, fachliche Einweisung und Unterstützung bei den experimentellen Arbeiten (Induktion einer retinale Ischämie, Gewebeaufbereitung), fachlich-kritische Diskussionen, Unterstützung bei der Erstellung der Manuskriptendversion.

Prof. Dr. med. Otto W. Witte

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